

## Tracking the Source of the Hepatitis B Virus–Specific CD8 T Cells during Lamivudine Treatment

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**Lamivudine treatment in chronic hepatitis B leads to the reconstitution of virus-specific T cells in the circulation, but it is not clear whether this is the preferential result of T cell efflux from the liver or lymph nodes. To address this question, the frequency and function of liver-, lymph node-, and blood-derived hepatitis B virus (HBV)–specific CD8 T cells were analyzed in patients treated with lamivudine and undergoing liver transplantation. HBV-specific CD8 T cells, identified in portal lymph nodes, were able to expand in vitro after antigen-specific stimulation and displayed a heterogeneous profile of cytokine production. These findings suggest that the peripherally reconstituted HBV-specific CD8 T cells can originate from precursor cells within lymph nodes.**

The expansion of a hepatitis B virus (HBV)–specific T cell response to the levels found in patients who recover from the infection represents a goal in the treatment of patients with chronic hepatitis B [1]. Immunotherapeutic strategy [2–4] designed to boost the weak virus-specific T cell response present in chronically infected patients requires the presence of a population of cells that are not terminally differentiated [5]. The

inhibition of viral replication by use of the nucleoside analogue lamivudine results in an increase frequency of circulating HBV-specific T cells [6, 7]. Yet, it has not been possible to determine whether this reconstitution is derived from an efflux of terminally differentiated liver-derived T cells or from a T cell population present in secondary lymphoid organs that have a potential for further expansion. To address this question, we studied peripheral blood (PB)–, liver–, and lymph node (LN)–derived HBV-specific CD8 T cells in patients with chronic hepatitis B during lamivudine treatment.

**Patients, materials, and methods.** Two HLA-A2–positive patients with chronic hepatitis B were studied (patient A: hepatitis B surface antigen [HBsAg] positive; anti-HBe positive; HBV-DNA load 92,000 copies/mL; and alanine aminotransferase [ALT] level, 80 U/L; patient B: HBsAg positive; anti-HBe positive; HBV-DNA load 82,000 copies/mL; and ALT level, 52 U/L). Lamivudine treatment was begun 3 months before liver transplantation for focal hepatocellular carcinoma. For patient A, liver and blood samples were obtained before lamivudine treatment and at the time of transplantation. A liver biopsy sample with nontumor area was obtained before lamivudine treatment. This liver tissue sample was divided into 2 parts: one for histological examination and the other for our research. Histological examination showed chronic hepatitis without cirrhosis. At the time of transplantation, a 2-cm<sup>3</sup> sample of apparently nontumor liver and a portal LN were obtained from both patients. A portal LN from an HLA-A2–positive non-HBV–infected patient (transplanted for hepatitis C) was obtained as a control.

Peptides corresponding to the sequence of HBV genotype D were purchased from Primm. Purity was >90%. Soluble HLA-A2 peptide tetramers were produced, as described elsewhere [8], and were bought from Proimmune. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by gradient centrifugation on ficoll-hypaque. Mononuclear cells were purified from biopsy specimens, according to methods described elsewhere [9]. LNs were homogenized mechanically in a petri dish, suspended in RPMI 1640 medium, and mononuclear cells isolated on ficoll-hypaque. PB- and LN-derived cells ( $0.5\text{--}1 \times 10^6$ ) were incubated for 30 min at 37°C with 1  $\mu\text{g}/\text{mL}$  tetramers, were washed in PBS, and then were stained with anti-CD8/Cy-chrome antibody. After washing, cells were analyzed on a Becton Dickinson FACS by use of CELLQuest software (Becton Dickinson). For PB- and LN-derived cells,  $\sim 0.4 \times 10^6$  cells were acquired within the live gate, to ensure that at least  $0.05 \times 10^6$  CD8 T cells were available for analysis.

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Informed consent was obtained from patients, and human experimentation guidelines of the US Department of Health and Human Services and those of the authors' institutions were followed. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

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**Table 1. Direct ex vivo frequency of hepatitis B virus tetramer-positive CD8 T cells (patient A).**

Anatomical site	HLA-A2 tetramer			
	Tc18-27	Tp575-83	Te183-91	Te348-57
Before therapy				
Blood	0.005	0.01	0.01	0.01
Liver	0.01	ND	ND	ND
During therapy				
Blood	<b>0.08</b>	0.02	0.03	0.01
Liver	<b>0.11</b>	0.03	0.02	0.03
Lymph node	<b>0.11</b>	<b>0.04</b>	<b>0.04</b>	<b>0.04</b>

**NOTE.** Data are percentage of frequency of CD8<sup>+</sup> cells found in the indicated anatomical sites that are positive for the indicated HLA tetramers. Boldface type indicates frequencies above the background level found in controls. ND, not done.

In the study of liver-infiltrating tetramer-positive cells, all the cells available from a given biopsy sample were processed and acquired, to allow the analysis of at least  $3 \times 10^4$  CD8 T cells within the live gate.

PBMC were suspended at a concentration  $3 \times 10^6$ /mL in RPMI 1640 medium and 10% fetal calf serum. Cells were stimulated with 1  $\mu$ M of different peptides in a 96-well plate. Recombinant interleukin (IL)-2 (50 IU/mL) was added on day 4 of culture, and cells were analyzed after 10–12 days of culture.

Short-term T cell lines were stimulated at  $2-3 \times 10^6$ /mL with or without peptides for 6 h at 37°C in the presence of 10  $\mu$ g/mL brefeldin A. Cells were washed, stained with Cy-chrome-conjugated anti-CD8, and then permeabilized and fixed by use of Permeafix (Ortho Diagnostic Systems), according to the manufacturer's instructions. Fluorescein isothiocyanate-conjugated anti-cytokine antibody (IFN- $\gamma$ , IL-4, or IL-2) or isotype-matched control was added (for 30 min at 4°C), and the mixture was washed twice and analyzed by flow cytometry.

**Results.** The direct ex vivo frequency of HBV-specific CD8 T cells in PB and liver was tested before and during therapy in patient A, with HLA-A2 peptide tetrameric complexes specific for HBV core 18-27 (Tc18-27), envelope 183-91 (Te183-91), envelope 348-57 (Te348-57), and polymerase 816-24 (Tp816-24). The frequency of tetramer-positive cells specific for envelope and polymerase epitopes in the circulation before and during lamivudine treatment was lower than the background level (0.05% of total CD8 T cells) [8]. An increase in the frequency of HBV-specific CD8 T cells in the circulation during treatment was seen only for Tc18-27-positive cells (0.08% vs. 0.005% before therapy; table 1).

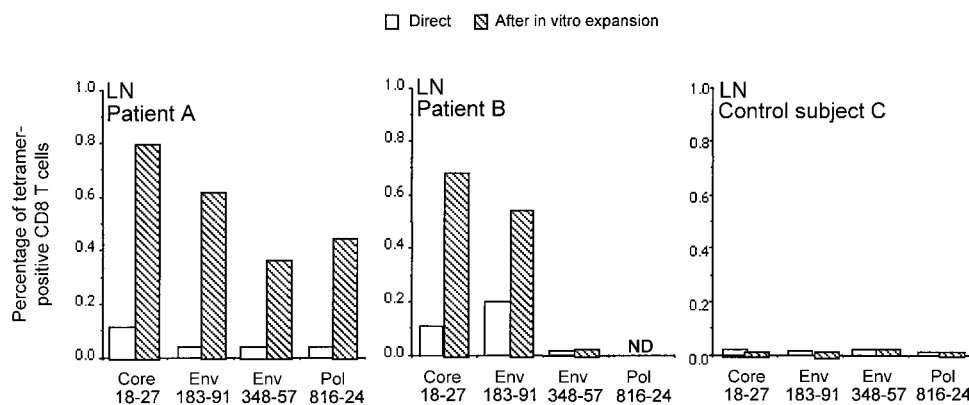
The limited numbers of intrahepatic cells obtained from the pretransplant liver biopsy ( $\sim 0.4 \times 10^6$  cells) allowed for only the direct analysis of core 18-27-specific CD8 T cells. Tc18-27 + CD8 T cells were not detectable in the liver before therapy, but this specificity accounted for 0.11% of intrahepatic CD8 T cells during treatment (table 1). The absence of Tc18-27 + CD8 T cells

in the liver before lamivudine treatment suggests that their increase in the circulation during therapy was not due to an efflux from the infected liver. The frequency of tetramer-positive cells specific for envelope and polymerase epitopes in the liver was lower than the background level (0.04% of total CD8 T cells).

Direct staining of cells isolated from the portal LN of patient A demonstrated a clear population of Tc18-27 + CD8 T cells with a frequency similar to that seen in the liver and blood (table 1). LN-derived CD8<sup>+</sup> T cells were visualized directly ex vivo by tetramers Te183-91, Te348-57, and Tp816-24 at a frequency of 0.04% above the background level (0.01%–0.02%) found in cells derived from the LN of an HLA-A2-positive non-HBV-infected subject (control subject C; figure 1). The presence of CD8 T cells specific for core 18-27, envelope 183-91, envelope 348-57, and polymerase 816-24 in the LN of patient A was confirmed by stimulating LN-derived cells with HBV peptides. HBV-specific CD8 T cells clearly expanded from the LN cells of patient A but not from peptide-stimulated LN cells from control subject C (figure 1). In contrast to LN-derived HBV-specific CD8 cells, the circulating core 18-27-specific cells expanded only after repetitive rounds of in vitro re-stimulation (data not shown).

The presence of an LN-derived population of HBV-specific CD8 T cells was confirmed in a second patient with chronic hepatitis B (patient B; figure 1). An analysis of HBV-specific CD8 T cell frequency was performed in this patient during lamivudine treatment only. Direct ex vivo staining of liver and LN-derived cells confirmed the presence of HBV-specific CD8 T cells at these sites (Tc18-27 CD8 T cells, liver 0.12% and LN 0.13%; Te183-91 CD8 T cells, liver 0.3% and LN 0.22%). Also in patient B, LN-derived core 18-27- and envelope 183-91-specific CD8 T cells efficiently expanded in vitro after peptide specific stimulation (figure 1).

Visualization of HBV-specific CD8 T cells with tetramers allows for their frequency to be defined but does not provide information about function. The cytokine production of the LN-derived HBV-specific CD8 T cells of both patients was analyzed after peptide in vitro stimulation. We chose this strategy because, although in vitro growth could potentially alter the initial cytokine profile, the low number of cells and the low frequency of antigen-specific CD8 T cells found directly with tetramers did not allow for us to perform direct ex vivo cytokine production analysis. In patient A, polymerase 455-63-specific CD8 T cells produced IFN- $\gamma$ , whereas core 18-27- and envelope 183-91-specific CD8 T cells produced only IL-2 and IL-4 (figure 2). The heterogeneous profile of cytokines production present in the HBV-specific CD8 T cells of patient A (polymerase-specific CD8 T cells/IFN- $\gamma$  and core-envelope-specific CD8 T cells/IL-4) was confirmed in patient B, in whom LN-derived Tc18-27-positive CD8 cells were classic Tc1 cells producing only IFN- $\gamma$  (figure 2). Of interest, after 2 rounds of in vitro



**Figure 1.** Direct frequency and expansion ability of lymph node (LN)-derived hepatitis B virus-specific CD8 T cells. LN cells were stained with the indicated tetramers. Frequencies of tetramer-positive cells directly and after 10 days of stimulation with the corresponding peptides were calculated by counting tetramer-positive cells out of CD8 T cells by fluorescence-activated cell sorting within the live gate. Env, envelope; ND, not done; Pol, polymerase.

stimulation, IL-4-producing CD8 T cells from patient A switched to (or were outgrown by) classic IFN- $\gamma$ -producing CD8 T cells (data not shown). Collectively, these data suggest that the cytokine production of LN-derived HBV-specific CD8 cells is highly heterogeneous, but in both patients these cells can be driven to produce antiviral cytokine (IFN- $\gamma$ ).

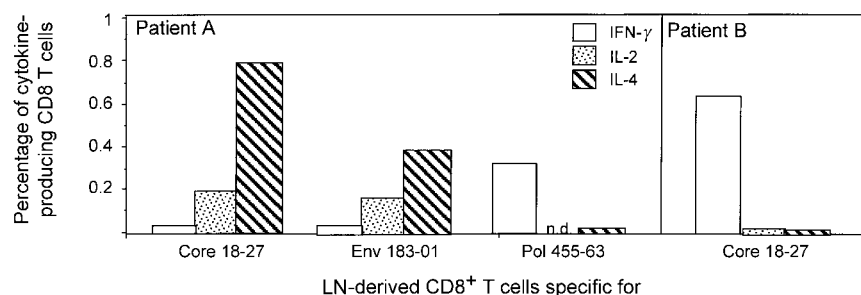
**Discussion.** For the present report, we analyzed whether the peripheral reconstitution of HBV-specific CD8 T cells observed in patients with chronic hepatitis B during lamivudine treatment is derived from an efflux of HBV-specific T cells from the infected liver or from a precursor T cell population within secondary lymphoid organs.

Two distinct pieces of evidence support the latter explanation. First, in patient A, we observed an increased frequency of circulating core 18-27-specific CD8 T cells during lamivudine therapy, despite these cells not being present within the liver before therapy. This observation cannot be reconciled with an efflux of HBV-specific CD8 cells from the liver during therapy. Second, multispecific HBV-specific CD8 T cells can be

demonstrated in the LN of patients with chronic hepatitis B during lamivudine treatment.

These data do not exclude that, in different clinical situations, circulating HBV-specific CD8 cells also might derive from intrahepatic HBV-specific CD8 T cells. A low frequency of HBV-specific CD8 T cells is present within the liver [10], and these cells can be expanded in vitro [11]. Nevertheless, the demonstration that the peripheral repertoire of HBV-specific CD8 T cells can be reconstituted with cells derived from secondary lymphoid organs has practical implications. The LN-derived HBV-specific CD8 T cells can reconstitute specificities that were not apparently present before therapy (patient A), and these cells, in contrast to liver- and blood-derived HBV-specific CD8 cells, efficiently expanded in vitro. This indicates that these cells are not terminally differentiated and supports the possibility of boosting HBV-specific T cell response in vivo with the use of therapeutic immunization.

Because of the essential role of IFN- $\gamma$  [12] in the clearance of HBV-infected hepatocytes, we analyzed the cytokine-pro-



**Figure 2.** Specific cytokine production of lymph node (LN)-derived hepatitis B virus-specific CD8 T cells from patients A and B. Polyclonal cell lines were produced by short-term (10 days) stimulation of LN-derived cells with the indicated peptides. Bars represent the percentage of peptide-specific cytokines producing CD8 T cells, calculated as follows: [(peptide stimulated cytokines + CD8 T cells/total CD8 T cells - nonstimulated cytokines + CD8 T cells)/total CD8 T cells]  $\times$  100. Env, envelope; IFN, interferon; IL, interleukin; Pol, polymerase.

duction profile of the LN-derived T cell population. Our data, although derived from a very limited number of HBV-infected subjects, showed that LN-derived HBV-specific CD8 T cells present a heterogeneous profile of cytokine production. In patient B, HBV-specific CD8 T cells were classical Tc1 cells producing IFN- $\gamma$ . In contrast, core- and envelope-specific CD8 T cells of patient A displayed a Th0-Th2 cytokine profile, as seen in other patients with chronic hepatitis B [9, 13, 14]. A further in vitro round of stimulation of LN cells led to the appearance of Tc1 CD8 T cells, but the extent to which multiple rounds of in vitro stimulation might mimic in vivo events is uncertain.

In conclusion, immunologic parameters were analyzed in different anatomical sites in 2 patients with chronic hepatitis B treated with lamivudine. The reconstitution of a circulating population of HBV-specific CD8 T cells did not seem to result from the efflux of cells from the liver but derived mainly from a population of cells present in secondary lymphoid organs. Peptide stimulation of LN-derived cells expanded HBV-specific CD8 T cells, with a heterogeneous profile of cytokine expression, which, after repeated in vitro stimulation, produced IFN- $\gamma$ . These results support the possibility that therapeutic immunization in patients treated with antiviral drugs might allow for further expansion of a potentially effective antiviral CD8 T cell response.

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