

Quantitative and functional analysis of PDC-E2-specific autoreactive cytotoxic T lymphocytes in primary biliary cirrhosis

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While the pathologic mechanisms responsible for organ-specific tissue damage in primary biliary cirrhosis (PBC) remain an enigma, it has been suggested that the pathology is mediated by autoreactive T cells infiltrating the intrahepatic bile ducts. Previously, we have documented that there is 100-fold enrichment in the frequency of CD4⁺ autoreactive T cells in the liver that are specific for peptides encoded by the E2 components of the pyruvate dehydrogenase complexes (PDC-E2). We have also recently characterized the first MHC class I-restricted epitope for PDC-E2, namely amino acid 159–167, a region very similar to the epitope recognized by MHC class II-restricted CD4⁺ cells and by autoantibodies. The effector functions of these PDC-E2₁₅₉₋₁₆₇-specific CD8⁺ cytotoxic T lymphocytes (CTLs) are not well understood. We have taken advantage of tetramer technology and report herein that there is tenfold increase in the frequency of PDC-E2₁₅₉₋₁₆₇-specific CTLs in the liver as compared with the blood in PBC. In addition, the precursor frequency of the CTLs in blood was significantly higher in early-stage PBC. Of interest was the fact that, upon stimulation with the peptide, the response of PDC-E2₁₅₉₋₁₆₇ tetramer-positive cells is heterogeneous with respect to IFN- γ synthesis. These data, we believe for the first time, document the enrichment of autoantigen-specific CD8⁺ T cells in the PBC liver, suggesting that CD8⁺ T cells play a significant role in the immunopathogenesis of PBC.

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Introduction

Primary biliary cirrhosis (PBC) is an autoimmune cholestatic liver disease characterized by the presence of antimitochondrial Ab's (AMAs) and intense biliary inflammatory response. The major mitochondrial antigen recognized by AMAs has been defined as the E2 component of the pyruvate dehydrogenase complex (PDC-E2) (1). Several lines of evidence suggest that T cells are implicated in the pathogenesis of PBC (2). This is exemplified by the presence of CD4⁺ and CD8⁺ T cell infiltrates within the portal tracts of the liver in PBC patients (3). MHC class II-restricted autoreactive CD4⁺ T cells specific for PDC-E2 have been identified from both peripheral blood and liver and the immunodominant target epitope has been defined to be amino acids 163-176 of PDC-E2 (4, 5). Moreover, we have recently identified an MHC class I-restricted CD8⁺ epitope of PDC-E2. This peptide, amino acid 159-167 of PDC-E2, can specifically induce CD8⁺ cytotoxic T lymphocyte (CTL) lines from the PBMCs of HLA-A*0201⁺ patients with PBC (6). These data suggest that much of the T cell and the autoantibody responses in this disease are directed against PDC-E2.

Although autoreactive CD8⁺ T cells are thought to be involved in the pathogenesis of several autoimmune diseases (7–9), the role of PDC-E2-specific CD8⁺ T cells in the pathogenesis of PBC remains elusive. Previous investigations have demonstrated the accumulation of antigen-reactive T cells at the site of the inflammation in several human autoimmune diseases as well as in murine models of human autoimmune diseases (9–12). It was thus reasoned that a comparative analysis of the precursor frequencies of PDC-E2-specific T cells isolated from the peripheral blood and from the liver of PBC patients would provide important information as to the relevance of such T cells in the pathogenesis of disease. In a previous report from our laboratory, 100-fold increase in the precursor frequency of PDC-E2-specific CD4⁺ T cells in the hilar lymph nodes and liver compared with PBMCs from patients with PBC has been documented (13). The recent advances in the identification of antigen-specific T cells with peptide-MHC tetramers, coupled with our identification of a HLA-A*0201-restricted immunodominant MHC class I epitope in PDC-E2, prompted us to use this technique

and knowledge to determine whether there is a similar selective enrichment of autoreactive MHC class I-restricted CD8⁺ CTLs in the diseased liver.

Materials

Patients and cells. The present study involved blood from 18 HLA-A*0201⁺ patients with PBC (PBC 1 to PBC 18; four at stage I, five at stage II, four at stage III, and five at stage IV), four HLA-A*0201⁺ healthy controls (normal 1 to 4), and four HLA-A*0201⁺ patients with other chronic liver diseases (chronic liver disease 1 to 4; three alcoholic hepatitis and one granulomatous hepatitis), and four HLA-A2⁻ patients with PBC (A2 negative PBC 25 to 28). The HLA-A*0201 haplotype of the subjects was determined initially by using HLA-A2-specific mAbs MA2.1 and BB7.2 as described previously (14) and confirmed by standard molecular MHC class I typing. PBMCs were purified from venous blood using standard Ficoll-Histopaque gradient centrifugation techniques. Liver-infiltrating lymphocytes (LILs) were harvested from explanted liver tissue of six HLA-A*0201⁺ PBC patients undergoing liver transplantation (PBC 19 to PBC 24), as described (13). LILs were also harvested from excess liver tissue of two HLA-A*0201⁺ normal adult livers donated for transplantation (normal 5 to 6). The PBMCs and LILs were cryopreserved in 10% DMSO/90% FCS and stored in liquid nitrogen until used.

Synthetic peptides. A synthetic peptide corresponding to the previously defined immunodominant HLA-A*0201-restricted CTL epitope of the autoantigen, amino acid 159-167 of the PDC-E2 (PDC-E2₁₅₉₋₁₆₇), was prepared (6). A previously defined HLA-A*0201-restricted CTL epitope, amino acid 18-27 of the hepatitis B virus core protein (HBC₁₈₋₂₇) (15), and amino acid 58-66 of influenza matrix protein (Flu MP₅₈₋₆₆) (16) were also used as negative controls. All peptides were synthesized with a free NH₂ and a free COOH terminus and their purity confirmed by HPLC. Lyophilized peptides were reconstituted at 10 mg/ml in DMSO and diluted in the appropriate medium before use.

Synthesis of peptide-MHC tetramers. PDC-E2₁₅₉₋₁₆₇-HLA A2 tetramers were generated as described (17). In brief, recombinant HLA-A*0201 with a 15-amino acid substrate peptide for BirA-dependent biotinylation at its C terminus was expressed in *Escherichia coli* BL21(DE3)pLysS (Promega Corp., Madison, Wisconsin, USA) carrying pET-HLA-A*0201 plasmid (Promega Corp.) and isolated from inclusion bodies. Recombinant β 2 microglobulin was also expressed in the same way using a plasmid carrying β 2 microglobulin (pHN1- β 2m), which was a gift from D.C. Wiley (Harvard University, Cambridge, Massachusetts, USA). The inclusion bodies were purified and dissolved in a urea-denaturing buffer. The monomeric MHC-peptide complexes were formed by combining the recombinant HLA-A*0201, recombinant β 2-microglobulin, and the nonapeptide PDC-E2₁₅₉₋₁₆₇ in an arginine-folding buffer. HLA-A*0201 was folded in the presence of β 2-microglobulin and a specific peptide to form a peptide-MHC complex,

following the procedure of Garboczi et al. (16). The complex was purified using a Sephacryl S100 column (Pharmacia Biotech Inc., Piscataway, New Jersey, USA). The MHC-peptide complexes were biotinylated enzymatically with BirA enzyme (Avidity, Denver, Colorado, USA), mixed with phycoerythrin-labeled Extraavidin (Sigma Chemical Co., Saint Louis, Missouri, USA) at a molar ratio of 4:1 to form the tetrameric peptide-MHC complex. Similarly prepared Flu MP₅₈₋₆₆-HLA-A*0201 tetramers were used as controls.

Generation of peptide-induced CTL lines. Peptide-specific CTL lines were generated as described previously (6). Dendritic cells (DCs) were generated from PBMCs of HLA-A*0201-positive donors as described (18) and used as antigen-presenting cells (APCs). In brief, PBMCs were resuspended in RPMI-1640 (Life Technologies Inc., Grand Island, New York, USA) supplemented with 10% heat-inactivated FCS, penicillin (50 U/ml), and streptomycin (50 μ g/ml) (FCS medium), seeded in a 250-ml culture flask, and incubated at 37°C for 1.5 hours. After removing nonadherent cells in the medium, the adherent cells were fed with FCS medium supplemented with GM-CSF (1,000 U/ml) (PreproTech Inc., Rocky Hill, New Jersey, USA) and IL-4 (1,000 U/ml) (PreproTech Inc.) and cultured at 37°C. On day 7, cells were harvested and incubated overnight in FCS medium containing PDC-E2₁₅₉₋₁₆₇ peptide (10 μ M). The cells were then washed and γ irradiated (5,000 Gy). Cryopreserved PBMCs or LILs were thawed, washed twice with HBSS, resuspended in FCS medium, and seeded in 24-well plates at 2×10^6 cells per well along with 2×10^5 peptide-pulsed APCs. Cells were cultured at 37°C for a total of 14 days, with appropriate feeding of the cultures with media. On day three, recombinant human IL-2 (Pepro-Tech) was added to each well at a final concentration of 10 U/ml. In select experiments, PBMCs or LILs were labeled with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes Inc., Eugene, Oregon, USA) prior to coculture with APCs. In these experiments the cryopreserved cells were thawed, washed twice with serum-free PBS, and labeled with 0.5 μ M CFSE for 10 minutes at 37°C. The labeling was terminated by the addition of FCS, and subsequently the cells were washed six times with FCS medium. The CFSE-stained cells were seeded in 24-well plates at 2×10^6 cells per well, along with 2×10^5 peptide-pulsed APCs, and cultured for 14 days as described above.

Flow-cytometric analysis and cell sorting. Peptide-specific CTL lines were prepared as described above, resuspended in 50 μ l of FACS buffer (0.5% BSA and 0.05% sodium azide in PBS), and incubated at 4°C with Fc γ receptor Ab (Miltenyi Biotec, Auburn, California, USA) and NeutrAvidin (Molecular Probes Inc.) for 15 minutes. Cells were stained with the appropriate peptide-loaded PE-labeled HLA-A*0201 tetramer for 30 minutes at room temperature in the dark, washed, and subsequently incubated with FITC-labeled anti-CD8 Ab's (Caltag Laboratories Inc., Burlingame, California, USA) and tri-color (TC)-labeled anti-CD4 Ab's (Caltag

Laboratories Inc.) for 30 minutes at 4°C in the dark. After incubation, the cells were washed twice with FACS buffer, fixed with 1% paraformaldehyde in PBS, and analyzed by three-color flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). The acquired data were analyzed with CELLQUEST software (BD Biosciences, San Jose, California, USA). In some experiments, both tetramer-positive and negative populations were sorted with a MoFlo cell sorter (Cytomation Inc., Fort Collins, Colorado, USA).

Intracellular cytokine analysis using flow cytometry. Peptide-induced CTL lines were incubated in fresh FCS medium with peptide (10 µM) and brefeldin A (10 µg/ml) (Sigma Chemical Co.) at 37°C for 4 hours. Cells were harvested and treated first with FACS lysing solution and then with FACS permeabilizing solution (BD Biosciences). The processed cells were resuspended in 50 µl of FACS buffer and stained with PE-labeled anti-CD8 (Caltag Laboratories Inc.), TC-labeled anti-CD4 (Caltag Laboratories Inc.), and FITC-labeled Ab's against one of the following cytokines: IFN-γ, TNF-α, or IL-2 (BD Biosciences). An aliquot of the cells was also stained with FITC-labeled anti-CD8 (Caltag Laboratories Inc.), TC-labeled anti-CD4 (Caltag Laboratories Inc.), and PE-labeled Ab's against one of the following cytokines: IL-4 or IL-10 (BD Biosciences). In some experiments, IFN-γ production of tetramer-binding cells was directly assessed by a combination of tetramer staining and intracellular staining for IFN-γ, as described (14, 19), with some modifications. The cells were incubated in fresh FCS medium with peptide (10 µM) and brefeldin A (10 µg/ml) at 37°C for 4 hours. Cells were harvested and resuspended in 50 µl of FACS buffer and stained with PE-labeled tetramer for 30 minutes at room temperature in the dark, washed, and subsequently treated with FACS lysing solution and FACS permeabilizing solution. The processed cells were stained with FITC-labeled anti-IFN-γ Ab's and TC-labeled anti-CD8 Ab's as described above. The stained cells were analyzed on a FACScan flow cytometer with CELLQUEST software (BD Biosciences).

Cytotoxicity assay. The HLA-A*0201⁺ lymphoblastoid T2 cell line was pulsed with appropriate peptides (10 µM) at 37°C overnight and used as target cells. To assess the antigen-specific cytolytic activity of the CTL lines, a fluorescence-based cytotoxicity assay was carried out with DELFIA EuTDA cytotoxicity assay reagents (Wallac Oy, Turku, Finland), as described (6). In brief, the peptide-pulsed target cells were washed, labeled with TDA (2,2':6',2''-terpyridine-6,6''-dicarboxylic acid), washed again, and resuspended in FCS medium. Five thousand target cells in a volume of 0.1 ml were plated into each well of a 96-well round-bottom plate, followed by the addition of varying numbers of effector cells in 0.1 ml of medium. After a 4-hour incubation at 37°C, the plates were centrifuged and a 20-µl portion of the supernatant from each well was transferred to a 96-well microtiter plate containing 200 µl of DELFIA europium

(Eu) solution in each well. The Eu forms a stable complex with released TDA in the mixture and generates fluorescence. The fluorescence of EuTDA was measured by a time-resolved fluorometer (1420 VECTOR; Wallac Oy). Percentage of cytotoxicity was calculated by the formula: $100 \times (\text{release in assay} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. Maximum release was determined by the lysis of target cells in triplicate wells with DELFIA lysis buffer (Wallac Oy). Spontaneous release was measured by incubating target cells in triplicate wells in the absence of effector cells. In all experiments, spontaneous release was less than 15% of maximum release. Results are reported herein as the mean of triplicate values.

Estimation of specific T cell frequency based on the number of cell division. The frequency of peptide-specific CD8⁺ T cells was analyzed as described previously (20), with some modification. In brief, an aliquot of CFSE-stained cells were suspended into the FCS medium and cultured at 37°C. On day 7, the cells were stimulated with 5 µM of phytohemagglutinin-P (PHA-P) and 10 U/ml of rIL-2 and further incubated at 37°C for another 7 days. Polyclonal stimulation of CFSE-stained cells with PHA-P and IL-2 results in cell division accompanied by 50% dilution of CFSE per cell division in the cells, allowing determination of the mean CFSE intensity of each generation. These values were used to calculate the average number of cell division(s) in cell cultures stimulated with peptides. Precursor frequency was estimated by dividing the number of amplified tetramer-positive cells by 2^x , where x is the average number of cell divisions, to determine the absolute number of tetramer-positive precursors and then dividing this value by the total number of cells analyzed.

Statistical analysis. Values were statistically analyzed using the unpaired *t* test.

Results

Peptide-loaded HLA-A*0201 tetramers bind specifically to HLA-A*0201-restricted CTLs. The specificity of tetramers binding to Ag-specific TCRs was verified using specific CTL lines induced by in vitro stimulation of PBMCs with peptide antigens. Aliquots of PBMCs from an HLA-A*0201⁺ PBC patient were cocultured with APCs pulsed with either PDC-E2₁₅₉₋₁₆₇ peptide or Flu MP₅₈₋₆₆ peptide for 14 days to expand peptide-specific CD8⁺ T cells in vitro. The peptide-specific CTL activity of the respective CTL lines was confirmed by a cytotoxicity assay (data not shown). Aliquots of these in vitro-expanded CTL lines were stained with either PE-labeled PDC-E2₁₅₉₋₁₆₇ tetramer or PE-labeled Flu MP₅₈₋₆₆ tetramer, along with FITC-labeled anti-CD8 Ab's and TC-labeled anti-CD4 Ab's and analyzed by flow cytometry (Figure 1). In the PDC-E2 peptide-primed CTL line, there was a distinct population (0.66%) of CD8⁺ T cells that stained positively with PDC-E2₁₅₉₋₁₆₇ tetramer, while no (<0.1%) Flu MP₅₈₋₆₆ tetramer-binding population was detected (Figure 1, a and c). On the

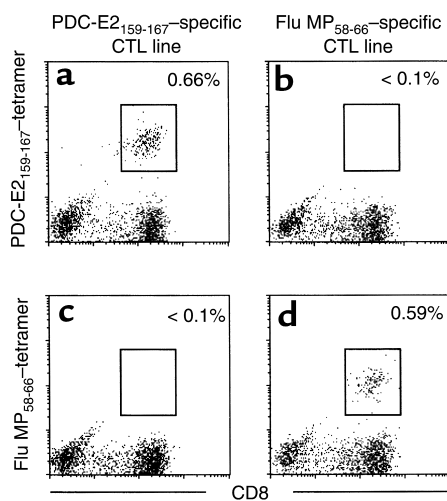


Figure 1
Verification of the binding specificity of peptide-MHC tetramers. PBMCs from an HLA-A*0201⁺ PBC patient were cocultured with PDC-E2₁₅₉₋₁₆₇-loaded APCs (a and c) or Flu MP₅₈₋₆₆-loaded APCs (b and d) for 14 days. The cells were stained with the HLA-A*0201 tetramer loaded with PDC-E2₁₅₉₋₁₆₇ (a and b) or the HLA-A*0201 tetramer loaded with Flu MP₅₈₋₆₆ (c and d) in addition to anti-CD4 and anti-CD8 Ab's, followed by flow cytometric analysis. Displayed in the dot plots are cells gated at lymphocyte population by forward scattering and side scattering and the CD4⁺ population. The cells within the box are considered positive. The number next to the box is the percentage of positively stained cells in the lymphocyte population.

other hand, in the Flu MP peptide-primed CTL lines, there was a distinct population (0.59%) of CD8⁺ T cells positively stained with Flu MP₅₈₋₆₆ tetramer, while no (<0.1%) binding was observed by the PDC-E2₁₅₉₋₁₆₇ tetramer (Figure 1, b and d). These experiments were repeated for both tetramers with independently generated CTL lines from six different HLA-A*0201⁺ individuals, which verified the binding specificity of the tetramers. The frequencies of the PDC-E2₁₅₉₋₁₆₇ tetramer-positive CD8⁺ cells after in vitro stimulation of PBMCs from 12 PBC patients with PDC-E2₁₅₉₋₁₆₇ are summarized in Table 1. It is important to note that the frequency of PDC-E2₁₅₉₋₁₆₇ tetramer-positive CD8⁺ cells were below the level of detection in the ex vivo-obtained PBMCs from all 12 PBC patients. The frequency of PDC-E2₁₅₉₋₁₆₇ tetramer-positive CD8⁺ cells after in vitro stimulation with PDC-E2₁₅₉₋₁₆₇ was below the level of detection in PBMCs from all 12 control individuals, including four HLA-A*0201⁺ healthy controls, four HLA-A*0201⁺ patients with other chronic liver diseases, and four HLA-A2⁻ patients with PBC (Table 1). The frequencies of Flu MP₅₈₋₆₆ tetramer-positive CD8⁺ cells after in vitro stimulation of PBMCs with Flu MP₅₈₋₆₆ peptide were between less than 0.1% and 10%, presumably reflecting a history of infection or immunization in each patient with PBC. The frequency of Flu MP₅₈₋₆₆ tetramer-positive CD8⁺ cells in the ex vivo-obtained PBMCs were between less than 0.1% and 0.2%, similar to the healthy donors in a previous report (21).

Effector functions of PDC-E2₁₅₉₋₁₆₇ peptide-induced CTL lines. To more fully characterize the autoreactive CTL lines specific for PDC-E2, a CTL line was prepared by stimulating PBMCs from an HLA-A*0201⁺ PBC patient with PDC-E2₁₅₉₋₁₆₇ peptide-pulsed APCs in vitro. The cells were split into aliquots and assessed for cytotoxicity, cytokine production, and tetramer binding, respectively. Target cells loaded with PDC-E2₁₅₉₋₁₆₇, but not with a control peptide, were lysed by the CTL lines at an effector/target (E/T) ratio as low as 10:1 (Figure 2a). When the cells were restimulated with PDC-E2₁₅₉₋₁₆₇ peptide in the presence of brefeldin A, followed by intracellular staining for IFN- γ , a distinct population (0.42%) of IFN- γ -producing cells was detected within the CD8⁺ T cell population (Figure 2b). The peptide-induced T cell lines were also stained with the PDC-E2₁₅₉₋₁₆₇ tetramer along with mAb to CD8 and CD4 and analyzed by flow cytometry. A distinct tetramer-positive CD8⁺ population (0.82%) was detected in PDC-E2 peptide-specific CTL lines (Figure 2c). The frequencies of the tetramer-positive CD8⁺ cells, IFN- γ -producing CD8⁺ cells, and percentage of cytotoxicity of the CTL lines from eight HLA-A*0201⁺ patients with PBC are summarized in Tables 1 and 2. The frequency of the tetramer-positive cells was always higher than that of the IFN- γ -producing cells in each of the CTL lines studied.

Specific cytotoxicity of the PDC-E2₁₅₉₋₁₆₇ tetramer-reactive cells. In efforts to confirm that the tetramer-reactive cells represent true MHC class I-restricted autoreactive cytotoxic T cells, highly enriched populations of both PDC-E2₁₅₉₋₁₆₇ tetramer-positive and -negative CD8⁺ T cells were obtained by flow-cytometric sorting from PDC-E2₁₅₉₋₁₆₇ peptide-induced CTL lines. The sorted cells were used as effector cells in a cytotoxicity assay.

Table 1
Tetramer staining of PDC-E2 specific CD8 T cells after in vitro stimulation of PBMCs with PDC-E2₁₅₉₋₁₆₇

Sample	PDC-E2 ₁₅₉₋₁₆₇ tetramer ⁺ CD8 ⁺ cells (%)	Sample	PDC-E2 ₁₅₉₋₁₆₇ tetramer ⁺ CD8 ⁺ cells (%)
PBC 2	0.52	CLD ^A 1	<0.1
PBC 3	1.13	CLD 2	<0.1
PBC 5	0.53	CLD 3	<0.1
PBC 8	0.13	CLD 4	<0.1
PBC 9	0.14	Normal 1	<0.1
PBC 10	0.24	Normal 2	<0.1
PBC 13	0.82	Normal 3	<0.1
PBC 14	0.36	Normal 4	<0.1
PBC 15	0.66	A2 neg PBC ^B 25	<0.1
PBC 16	0.27	A2 neg PBC 26	<0.1
PBC 17	0.12	A2 neg PBC 27	<0.1
PBC 18	1.20	A2 neg PBC 28	<0.1
Mean \pm SD	0.42 \pm 0.33 ^C		

^ACLD = chronic liver disease controls ^BA2 neg PBC = HLA A2 negative PBC ^CValues for PBC patients were significantly ($P < 0.0001$) higher than those from controls (CLD, Normals, and A2 neg PBC).

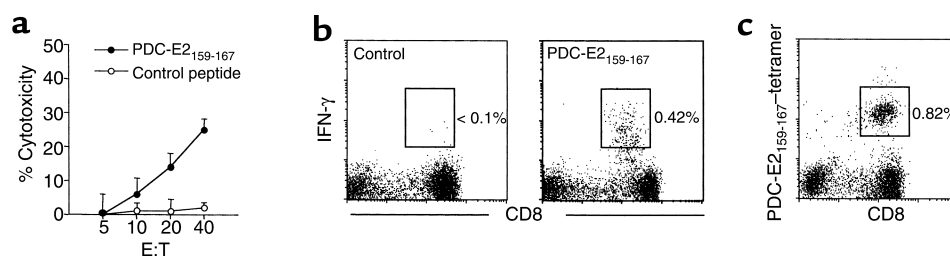


Figure 2

Identification and functional analysis of PDC-E2₁₅₉₋₁₆₇-specific CD8⁺ T cells in the peptide-induced CTL line. PBMCs from an HLA-A*0201⁺ PBC patient were cocultured with PDC-E2₁₅₉₋₁₆₇-loaded APCs for 14 days to generate a CTL line. (a) The CTL line was tested for cytotoxicity against PDC-E2₁₅₉₋₁₆₇ peptide- or control peptide-loaded T2 targets at different E/T ratios. HBC₁₈₋₂₇, an HLA-A*0201-restricted irrelevant epitope, was used as control. Displayed are the mean specific lysis of triplicate testing. (b) The CTL line was restimulated with PDC-E2₁₅₉₋₁₆₇ peptide or control peptide in the presence of brefeldin A, followed by intracellular staining for IFN-γ. Displayed in the dot plots are cells gated at lymphocyte population by forward scattering and side scattering and the CD4⁻ population. The number next to the box is the percentage of IFN-γ-producing cells in the lymphocyte population. (c) The CTL line was stained with PDC-E2₁₅₉₋₁₆₇ tetramer and Ab's against CD4 and CD8, followed by flow cytometric analysis. Displayed in the dot plots are cells gated at lymphocyte population by forward scattering and side scattering and the CD4⁻ population. The number next to the box is the percentage of positively stained cells in the lymphocyte population.

PDC-E2₁₅₉₋₁₆₇-specific lytic activity was detected only in the tetramer-positive population, not in the tetramer-negative population. Representative data from a PBC patient are shown in Figure 3. A highly enriched population of Flu MP₅₈₋₆₆ tetramer-positive CD8⁺ T cells, prepared in the same way from Flu MP₅₈₋₆₆ peptide-induced CTL lines, showed greater than 80% of Flu MP₅₈₋₆₆-specific lytic activity at an E/T ratio of 10 (data not shown).

Cytokine production of the PDC-E2₁₅₉₋₁₆₇ tetramer-reactive cells. The frequency of PDC-E2₁₅₉₋₁₆₇ tetramer-positive cells was constantly higher than IFN-γ-producing cells in response to restimulation with PDC-E2₁₅₉₋₁₆₇ peptide; this suggests the presence of PDC-E2₁₅₉₋₁₆₇ tetramer-reactive cells that do not respond with IFN-γ production after short-term restimulation with the PDC-E2₁₅₉₋₁₆₇ peptide. Thus, the ability of individual tetramer-binding cells to produce IFN-γ in response to the PDC-E2₁₅₉₋₁₆₇ peptide stimulation was assessed. PDC-E2₁₅₉₋₁₆₇-specific CTL lines from HLA-A*0201⁺ PBC patients were restimulated with PDC-E2₁₅₉₋₁₆₇ or a control peptide in the presence of brefeldin A, stained with the PDC-E2₁₅₉₋₁₆₇ tetramer, fixed and permeabilized, and stained for intracellular IFN-γ along with mAb against CD8. For purposes of control, aliquots of the same PBMCs were cocultured with APCs pulsed with Flu MP₅₈₋₆₆ peptide for 14 days to expand Flu MP₅₈₋₆₆ peptide-specific CD8⁺ T cells in vitro. When the Flu MP₅₈₋₆₆-specific CTL lines were restimulated with Flu MP₅₈₋₆₆ peptide, virtually all (> 99%) the Flu MP₅₈₋₆₆ tetramer-positive cells produced high levels of IFN-γ (Figure 4b). On the other hand, when the PDC-E2₁₅₉₋₁₆₇-specific CTL lines were stimulated with PDC-E2₁₅₉₋₁₆₇ peptide, two distinct tetramer-positive populations with different intensity of tetramer staining were identified. One of them was responsive to the specific peptide stimulation, as indicated by the downmodulated tetramer staining (14, 19) as well as production of IFN-γ. However, the level

of IFN-γ production by these cells was lower than that of the Flu MP₅₈₋₆₆ tetramer-positive cells. The other tetramer-positive population was nonresponsive to peptide stimulation, as indicated by the unchanged tetramer-staining intensity and the lack of IFN-γ production (Figure 4a). Similar results were reproduced in six patients with PBC (data not shown). These results suggest that the capability of IFN-γ production was lower and heterogeneous within the PDC-E2₁₅₉₋₁₆₇ tetramer-positive population. It is also important to note that the mean density of tetramer-positive cells for the Flu MP peptide was lower than the PDC-E2

Table 2

IFN-γ production and cytotoxicity of PDC-E2 specific CD8 T cells after in vitro stimulation of PBMCs with PDC-E2₁₅₉₋₁₆₇

Sample	IFN-γ producing CD8 ⁺ cells (%)	Specific cytotoxicity (%) (E: T = 40)
PBC 2	0.30	20
PBC 3	0.73	43
PBC 5	0.28	28
PBC 10	0.15	17
PBC 13	0.42	25
PBC 14	0.13	10
PBC 16	0.10	12
PBC 18	0.83	48
Mean ± SD	0.37 ± 0.27 ^A	25.38 ± 13.8 ^B
CLD ^C 1	< 0.1	< 5
CLD 2	< 0.1	< 5
Normal 1	< 0.1	< 5
Normal 2	< 0.1	< 5
A2 neg PBC ^D 25	< 0.1	< 5
A2 neg PBC 26	< 0.1	< 5

^AFrequencies of IFN-γ producing CD8 T cells in PBC patients were significantly ($P < 0.0001$) higher than those from controls (CLD, Normals, and A2 neg PBC).

^BValues of specific cytotoxicity in PBC patients were significantly ($P < 0.0001$) higher than those from controls (CLD, Normals, and A2 neg PBC).

^CCLD = chronic liver disease controls ^DA2 neg PBC = HLA A2 negative PBC.

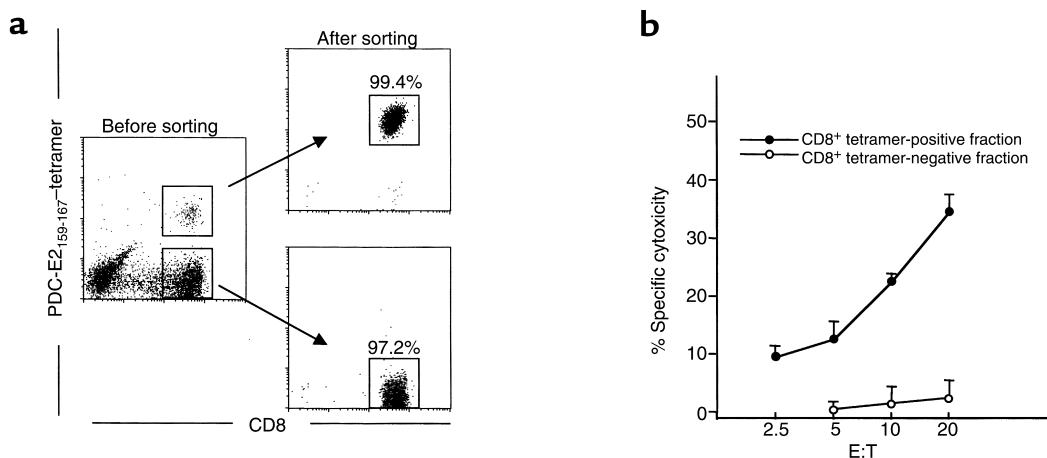


Figure 3

The specific cytotoxicity of PDC-E2₁₅₉₋₁₆₇ peptide-induced cell line is mediated by tetramer-positive CD8⁺ T cells. PBMCs from an HLA-A*0201⁺ PBC patient were cocultured with PDC-E2₁₅₉₋₁₆₇-loaded APCs for 14 days to generate a CTL line. (a) Cells were stained with the PDC-E2₁₅₉₋₁₆₇ tetramer and Ab's against CD4 and CD8, followed by flow cytometric cell sorting to isolate tetramer-positive CD8⁺ and tetramer-negative CD8⁺ populations. (b) The sorted cells were tested for their cytotoxicity against T2 target cells loaded with PDC-E2₁₅₉₋₁₆₇ peptide or a control peptide Hbc₁₈₋₂₇ at different E/T ratios. Displayed is the mean specific lysis of triplicate testing.

peptide-specific CD8⁺ T cells, denoting differences in the relative downregulation of TCRs in the two antigen-specific CTL lines. These data also suggest that part of the PDC-E2₁₅₉₋₁₆₇ tetramer-positive population is anergized. The ability of the PDC-E2₁₅₉₋₁₆₇ peptide-specific CTLs to produce other cytokines in response to the PDC-E2₁₅₉₋₁₆₇ peptide stimulation was also assessed. However, no detectable level of cells producing TNF- α , IL-2, IL-4, or IL-10 was observed in any of four patients studied (data not shown).

PDC-E2₁₅₉₋₁₆₇ tetramer-reactive cells in the liver. To assess whether PDC-E2₁₅₉₋₁₆₇-specific CD8⁺ T cells exist in end-stage PBC livers, LILs were isolated from explanted liver tissue of six HLA-A*0201⁺ patients (PBC 19 to 24) undergoing liver transplantation. There were undetectable levels of PDC-E2₁₅₉₋₁₆₇ tetramer-positive CD8⁺ T cells in uncultured LIL samples. However, when the LILs from the explanted livers of the PBC patients were stimulated with APCs pulsed with PDC-E2₁₅₉₋₁₆₇ peptide for 14 days to expand peptide-specific CD8⁺ T cells in vitro, tetramer-binding CD8⁺ T cells were readily detectable in each of the six PBC patients (Table 3). For comparison, LILs were isolated from the excess liver tissues donated by two healthy HLA-A*0201⁺ individuals (normal 5 to 6) intended for transplantation. These cells were also stimulated with APCs pulsed with PDC-E2₁₅₉₋₁₆₇ peptide for 14 days, followed by tetramer staining. No tetramer-positive CD8⁺ T cells were detected in the in vitro-cultured LILs from either donor (Table 3).

The frequency of PDC-E2₁₅₉₋₁₆₇ tetramer-reactive cells in the blood and liver. To estimate the frequency of PDC-E2₁₅₉₋₁₆₇-specific CD8⁺ T cells in the peripheral blood and liver, PBMCs from 12 HLA-A*0201⁺ patients with PBC at different stages (PBC 1 to 12) and LILs from end-stage PBC liver of six HLA-A*0201⁺ PBC

patients (PBC 19 to 24) were first stained with CFSE, then cocultured with APCs pulsed with PDC-E2₁₅₉₋₁₆₇ peptide for 14 days, to expand peptide-specific CD8⁺ T cells. The induced CTL lines were stained with PDC-E2₁₅₉₋₁₆₇ tetramer, along with mAb to CD8, and analyzed by flow cytometry. A distinct population of CD8⁺ T cells positively stained with the PDC-E2₁₅₉₋₁₆₇ tetramer was detected in 10 of 12 of PBMCs and each of the six cases of LILs studied. Representative results of a PBMC sample and a LIL sample are shown in Figure 5, a and b. The number of cell divisions undergone by the tetramer-positive cells was quantitated relative to the intensity of CFSE fluorescence and was similar in PBMCs and LILs, with values of 6.20 ± 0.79 compared with 6.50 ± 0.55 , respectively. The frequencies of the PDC-E2₁₅₉₋₁₆₇ tetramer-positive cells in the uncultured PBMCs were calculated as

Table 3

Tetramer staining of PDC-E2 specific CD8 T cells after in vitro stimulation of LILs with PDC-E2₁₅₉₋₁₆₇

Sample	PDC-E2 ₁₅₉₋₁₆₇ tetramer ⁺ CD8 ⁺ cells (%)
PBC 19	5.47
PBC 20	4.94
PBC 21	3.36
PBC 22	3.04
PBC 23	3.14
PBC 24	2.74
Mean \pm SD	3.78 \pm 1.13 ^A
Normal 5	< 0.1
Normal 6	< 0.1

^AValues for PBC patients were significantly ($P = 0.0047$) higher than those from controls

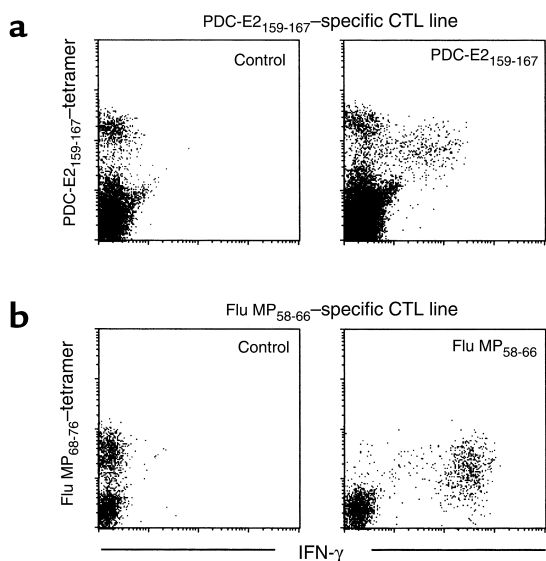


Figure 4

Antigen specificity of IFN- γ production by CD8⁺ T cells for PDC-E2 autoantigen or influenza viral antigen. (a) PDC-E2₁₅₉₋₁₆₇ peptide-induced CTL lines derived from a PBC patient was restimulated with PDC-E2₁₅₉₋₁₆₇ peptide or a control peptide in the presence of brefeldin A. The cells were first stained with the PDC-E2₁₅₉₋₁₆₇ tetramer, followed by intracellular staining for IFN- γ . Displayed in the dot plots are cells gated at lymphocyte population by forward scattering and side scattering and the CD8⁺ population. (b) Flu MP₅₈₋₆₆ peptide-induced CTL lines derived from the same PBC patient was restimulated with Flu MP₅₈₋₆₆ peptide or a control peptide in the presence of brefeldin A. The cells were first stained with the Flu MP₅₈₋₆₆ tetramer, followed by intracellular staining for IFN- γ . Displayed in the dot plots are cells gated at lymphocyte population by forward scattering and side scattering and the CD8⁺ population.

stages I and II of PBC ($6.75 \pm 2.21 \times 10^{-5}$) as compared with later stages III and IV ($1.31 \pm 1.33 \times 10^{-5}$, $P < 0.0003$).

Discussion

CTLs play a pivotal role in host defense against viral infections and tumor development. In addition, several lines of evidence suggest that autoreactive CTLs are involved in the pathogenesis of human autoimmune diseases as well as animal models of autoimmune diseases (22–26). In a previous study we demonstrated that PDC-E2₁₅₉₋₁₆₇-specific CTL lines can be induced from PBMCs in HLA-A*0201⁺ PBC patients (6). In the present study, we have further characterized the peptide-induced CTLs using a PDC-E2₁₅₉₋₁₆₇-HLA-A*0201 tetramer reagent. Our results indicate that a distinct population of PDC-E2₁₅₉₋₁₆₇ tetramer-reactive cells can be detected after in vitro stimulation of PBMCs from HLA-A*0201⁺ PBC patients, but not from controls. These tetramer-reactive cells demonstrate specific cytotoxicity against PDC-E2₁₅₉₋₁₆₇-pulsed target cells, as shown by CTL assays using purified tetramer-positive and -negative cells. The in vitro-expanded PDC-E2-specific CD8⁺ T cells synthesized IFN- γ but

described above. More than 90% of the tetramer-positive cells was identified within the single peak of the cell division in all cases studied. The results for 12 PBMC samples and six LIL samples from HLA-A2*0201⁺ PBC patients, as well as eight PBMC samples and two LIL samples from HLA-A2*0201⁺ control, are summarized in Table 4. The average frequency of PDC-E2₁₅₉₋₁₆₇ tetramer-positive CD8 T cells in peripheral blood from 12 PBC patients was $3.58 \pm 3.26 \times 10^{-5}$, while that in the six end-stage PBC liver was $4.14 \pm 0.95 \times 10^{-4}$, over tenfold and significantly higher than that in the peripheral blood ($P < 0.0001$) (Table 4). These data suggest that PDC-E2₁₅₉₋₁₆₇-specific CTLs are markedly enriched in the liver. In addition, it is important to note that the frequency of PDC-E2₁₅₉₋₁₆₇-specific CD8⁺ T cells from peripheral blood were significantly higher in earlier

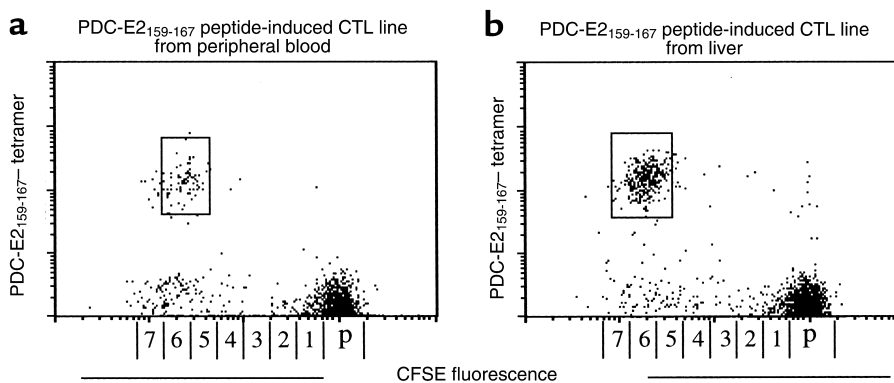


Figure 5

In vitro expansion of PDC-E2₁₅₉₋₁₆₇ tetramer-reactive cells. PBMCs (a) and LILs (b) from HLA-A*0201⁺ PBC patients were stained with CFSE and cocultured with PDC-E2₁₅₉₋₁₆₇-loaded APCs for 14 days. The cells were stained with the PDC-E2 tetramer and anti-CD8 Ab's, followed by flow cytometric analysis. Displayed in the dot plots are cells gated at lymphocyte population by forward scattering and side scattering and the CD8⁺ population. The number next to the box is the percentage of tetramer-stained cells in the lymphocyte population. The horizontal axis is labeled with numbers corresponding to cell divisions and with "p" depicting the undivided parent cell population. This scale was calculated from the distinct CFSE fluorescence peaks produced by polyclonal stimulation with PHA-P and IL-2.

Table 4
Precursor frequency of PDC-E2-specific CD8 T cells

Sample	Stage	Source	Frequency (per 100,000)	Average frequency (Mean ± SD)	Average frequency (Mean ± SD)
PBC 1	I	blood	4.68	^A 6.75 ± 2.21×10 ⁻⁵	^B 3.58 ± 3.26×10 ⁻⁵
PBC 2	I	blood	7.92		
PBC 3	I	blood	8.83		
PBC 4	II	blood	4.06		
PBC 5	II	blood	8.28		
PBC 6	III	blood	1.37		
PBC 7	III	blood	undetectable	^A 1.31 ± 1.33×10 ⁻⁵	
PBC 8	III	blood	2.10		
PBC 9	IV	blood	0.54		
PBC 10	IV	blood	3.75		
PBC 11	IV	blood	1.40		
PBC 12	IV	blood	undetectable		
CLD 1		blood	undetectable		
CLD 2		blood	undetectable		
CLD 3		blood	undetectable		
CLD 4		blood	undetectable		
Normal 1		blood	undetectable		
Normal 2		blood	undetectable		
Normal 3		blood	undetectable		
Normal 4		blood	undetectable		
PBC 19	IV	liver	42.70	^B 4.14 ± 0.95×10 ⁻⁴	
PBC 20	IV	liver	38.50		
PBC 21	IV	liver	52.50		
PBC 22	IV	liver	47.15		
PBC 23	IV	liver	24.53		
PBC 24	IV	liver	42.81		
Normal 5		liver	undetectable		
Normal 6		liver	undetectable		

^A*P* < 0.0003 comparing stage I and II to stage III and IV. ^B*P* < 0.0001 comparing blood to liver in patients with PBC.

not TNF- α and IL-2, the other two major type 1 cytokines produced by CD4⁺ Th1 cells and CD8⁺ CTLs specific for viral antigens. In addition, the synthesis of IL-4 and IL-10, major type 2 cytokines, were not observed in these cells.

In the present study, we demonstrate that the ability of the PDC-E2-specific CTLs detected by PDC-E2₁₅₉₋₁₆₇ tetramer to produce IFN- γ is lower and heterogeneous as compared with influenza-specific CTLs. One possible explanation is that some of the peptide-specific CD8⁺ T cells may lose their capability to synthesize cytokines during *in vitro* culturing. However, our finding that virtually all of the Flu MP₅₈₋₆₆-specific CD8⁺ T cells from the same patients were capable of producing IFN- γ argues against this explanation, suggesting that heterogeneity in cytokine profile may be an intrinsic character of the autoantigen-specific CD8⁺ T cells that is different from virus-specific CD8⁺ T cells. In contrast to virus-specific immunity, the immune response to tumor cells is often less vigorous and results in CTLs characterized by lower avidity and decreased functional capacity, especially when the tumor Ags are normal self proteins (27–29). Tumor-specific CTLs and autoreactive CTLs

may share common features because they both recognize self-antigen. In fact, it is plausible that the epitope derived from endogenous PDC-E2 could be expressed in association with the MHC molecule on all cells, even at a low level, which may lead autoreactive T cells to anergy. There is a relatively small increase in cytotoxicity by PDC-E2₁₅₉₋₁₆₇-specific CTLs after enrichment of the tetramer-positive cells, probably in part because of the modulation of the effector function of the cells by PDC-E2₁₅₉₋₁₆₇ tetramer directly bound on cells. The low lytic activity of highly enriched CD8⁺ cell line specific for PDC-E2₁₅₉₋₁₆₇, compared with highly enriched Flu MP₅₈₋₆₆-specific CTLs, may be explained by the functional impairment of the PDC-E2₁₅₉₋₁₆₇-specific CTLs.

An alternate interpretation of the functional impairment, in terms of cytokine production, is that the autoantigen-derived peptide-specific PDC-E2₁₅₉₋₁₆₇ is not identical to the epitope that induces the primary CD8⁺ T cell response, but a molecular mimic of the original immunogen. The primary CD8⁺ response may be elicited by an epitope of an unknown xenobiotic agent, such as a chemical or a virus (30). In this case, the

PDC-E2₁₅₉₋₁₆₇ epitope may actually be recognized by the xenobiotic-specific CD8⁺ T cells as an altered peptide ligand, which is known to be able to induce differential effector functions on the CD8⁺ T cells (31). This interpretation is consistent with the view that molecular mimicry may be operating in PBC.

Characterization of the intrahepatic T cell response is important for our understanding of the mechanisms underlying liver pathology. In the present study, PDC-E2₁₅₉₋₁₆₇-specific CTL lines have also been successfully induced from LILs of HLA-A*0201⁺ patients with end-stage PBC. By combination of CFSE staining and tetramer staining, we estimate that the frequency of PDC-E2₁₅₉₋₁₆₇-specific CD8 T cells in the end-stage PBC liver is 4.14×10^{-4} , while that in the periphery of PBC patients is 3.58×10^{-5} . These data suggest that PDC-E2₁₅₉₋₁₆₇-specific CD8⁺ T cells are enriched approximately tenfold in the liver. There is also the possibility that part of the PDC-E2₁₅₉₋₁₆₇-specific CTLs may stop dividing during in vitro culture due to anergy. However, most of the tetramer-positive cells were identified within a single peak of cell division, suggesting that PDC-E2₁₅₉₋₁₆₇-specific CTLs may undergo similar division. In an earlier study using limiting dilution analysis, the frequency of peripheral CD4⁺ helper T cells specific for the PDC-E2₁₆₃₋₁₇₆ epitope in PBC patients was estimated to be 3.66×10^{-7} , while that in the liver was 1.66×10^{-5} to 4.13×10^{-5} , 100-fold enrichment in the liver as compared with PBMC's (13). Taken together, these studies indicate that there is selective enrichment for both PDC-E2-specific CD4⁺ and CD8⁺ autoreactive T cells localized to the site of tissue injury in patients with PBC. The discrepancy of the level of frequency between autoreactive CD4⁺ and CD8⁺ T cells could be due to the difference of the methods of detection (32, 33). Interestingly, the extent of enrichment in the PDC-E2-specific autoreactive CD4⁺ T cells is higher than CD8⁺ T cells. This difference cannot be readily explained by the differences in the techniques used in the previous and present study. On the other hand, if indeed the values are, in fact, true differences, this could reflect a more important role for CD4⁺ T cells in the pathogenesis of PBC in late disease. Further study of CD4⁺ T cells using class II tetramer is warranted and may provide additional information about the nature of the autoreactive CD4⁺ T cells.

The estimated frequency of PDC-E2-specific CD8⁺ T cells from peripheral blood was significantly higher in early stages of PBC than in the more advanced disease stages. This is in agreement with a previous report showing that the frequency of peripheral blood CD4⁺ T cells that respond to the helper T cell epitope 163–176 was significant higher in the early stage of PBC as compared with the advanced stage (13). In previous studies directly comparing the frequency of hepatitis C virus-specific (HCV-specific) CD8⁺ T cells in peripheral blood and liver, higher frequencies of HCV-specific CD8⁺ T cells were found in liver biopsies than in the blood of patients with chronic HCV infection

(14, 34). In another study using end-stage cirrhotic liver, the HCV-specific T cell could not be found in the liver (35), suggesting that the level of virus-specific intrahepatic CTLs declines as the viral hepatitis progresses. Supposing that declination of the disease-specific CTL with the disease progression occurs in PBC, the PDC-E2-specific CD8⁺ T cells in the liver may be even more abundant during the earlier stages of PBC and be playing an important role in disease development. In addition, the autoreactive CTL response in PBC may also involve other epitopes restricted by HLA-A*0201, as well as epitopes restricted by other HLA molecules. Identification and characterization of these epitopes is required to generate a more complete picture of the overall CTL response in PBC during different disease stages.

In conclusion, this study provides evidence that PDC-E2-specific CD8⁺ T cells are enriched in the liver. In addition to our data on CD8⁺ T cells, previous studies have shown that high numbers of PDC-E2-specific CD4⁺ helper T cells, as well as B cells that produce PDC-E2 autoantibodies, are enriched in the PBC liver (13, 36). We believe that anti-PDC-E2 Ab's may form immune complexes with antigens and be taken up by APCs, including DCs (6). In addition, autoreactive B cells may be efficient APCs for priming T cells by specific uptake of the autoantigen via surface Ig receptor as reported in other autoimmune diseases (37, 38). These cells may lead to the activation of autoreactive CD4⁺ and CD8⁺ T cells, which in turn mediate damage on biliary epithelial cells (BECs) as well as activating autoreactive B cells to produce autoantibodies. Therefore the autoreactive B cells, CD4⁺ T cells, and CD8⁺ T cells may collectively form an orchestrated autoimmune effector response that leads to the pathogenesis of PBC. More importantly, the response is focused on PDC-E2, the major mitochondrial antigen, lending further weight to the argument that either native modified PDC-E2 and/or a mimeotope resembling PDC-E2 is instrumental in the etiology of disease.

Acknowledgments

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