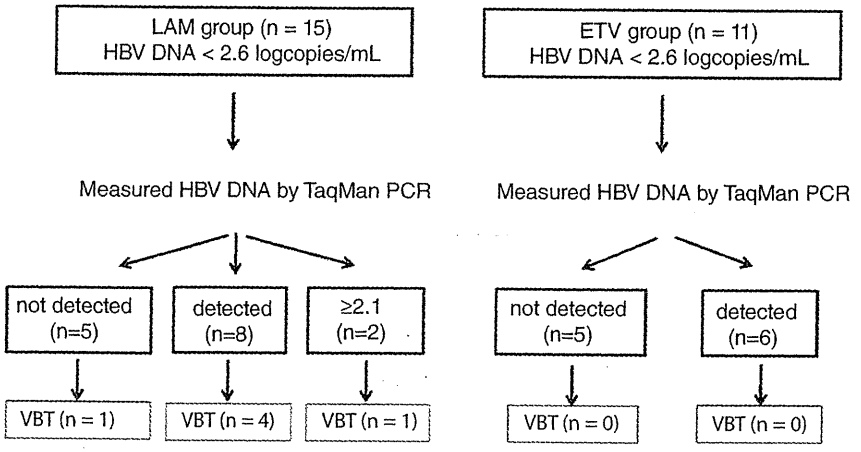

Figure 3 Incidence of virological breakthrough (VBT) based on the hepatitis B virus (HBV) DNA level at baseline by COBAS AmpliPrep-COBAS TaqMan HBV test (TaqMan PCR). The subsequent occurrence of VBT according to the DNA level by TaqMan PCR (not detected/detected/2.1 to <2.6 log copies/mL) was evaluated. In the lamivudine (LAM) group, VBT was observed in five of the 10 cases in which the results were either "detected" or ≥2.1 log copies/mL and in one of the five "not detected" cases. On the other hand, HBV DNA levels in the entecavir (ETV) group were "detected" in six, but there was no incidence of VBT.

five had no LAM resistance at baseline. However, the LAM resistance of rtM204V and rtL180M were found in all the patients with VBT in the LAM group. Moreover, a retrospective assessment by COBAS AmpliPrep-COBAS TaqMan HBV test showed that HBV DNA was detectable in 10 patients in the LAM group and six patients in the ETV group. Only five of the 10 patients in the LAM group had VBT, but none in the ETV group. In addition, one patient had VBT in the LAM group even though DNA was not detected by the TaqMan test, suggesting that switching to ETV was preferable. Hence, our data supported the 2010 Japanese guidelines which recommend switching to ETV in patients whose HBV DNA levels are less than 2.1 log copies/mL by TaqMan PCR.

A potential limitation of the present study is that the number of the cases was small. Nevertheless, our randomized controlled trial indicated significant difference in the incidence of VBT between the LAM and ETV groups. Therefore, this study is valuable for the purpose of verifying the 2007–2008 guidelines in Japan. In the present study, although no LAM-resistant mutant was observed in the ETV group at baseline, a very low level of LAM-resistant mutants may derive ETV resistance for long-term therapy. The results of switching to ETV in the present study were favorable during the 24-month observation period, but we have to be careful of possible emergence of ETV-resistant mutants in long-term follow up.

In conclusion, in patients treated with LAM for more than 3 years maintaining HBV DNA of less than 2.6 log copies/mL, switching treatment to ETV is recommended in at least a 2-year follow-up period.

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Fine Phenotypic and Functional Characterization of Effector Cluster of Differentiation 8 Positive T Cells in Human Patients With Primary Biliary Cirrhosis

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In primary biliary cirrhosis (PBC), patients develop a multilineage response to a highly restricted peptide of the E2 component of pyruvate dehydrogenase (PDC-E2) involving autoantibody and autoreactive cluster of differentiation (CD)4^+ and $CD8^+$ T-cell responses. Recent data from murine models have suggested that liver-infiltrating $CD8^+$ cells play a critical role in biliary destruction in PBC. We hypothesized that chronic antigen stimulation of $CD8^+$ T cells alters effector memory T cell (T_{EM}) frequency and function similar to that seen with chronic viral infections, including failure to terminally differentiate and relative resistance to apoptosis. We have rigorously phenotyped $CD8^+$ T-cell subpopulations from 132 subjects, including 76 patients with PBC and 56 controls, and report a higher frequency of T_{EM} cells characterized as $CD45RO^{high}CD57^+CD8^{high}$, but expressing the gut homing integrin, $\alpha4\beta7$, in peripheral blood mononuclear cells of PBC. These $CD8^{high}$ T_{EM} cells have reduced expression of Annexin V after TCR stimulation. Consistent with a T_{EM} phenotype, $CD45RO^{high}CD57^+CD8^{high}$ T cells express higher levels of granzyme A, granzyme B, perforin, CCR5 and $\alpha4\beta7$, and lower levels of CCR7 and CD28 than other $CD8^{high}$ T cells. Furthermore, interleukin (IL)-5 produced by $CD8^+CD57^+$ T lymphocytes upon *in vitro* T-cell receptor stimulation are increased in PBC. Histologically, $CD8^+CD57^+$ T cells accumulate around the portal area in PBC. Moreover, $CD8^+CD57^+$ T cells respond specifically to the major histocompatibility class I epitope of PDC-E2. *Conclusion:* In conclusion, our data demonstrate that $CD45RO^{high}CD57^+CD8^{high}$ T cells are a subset of terminally differentiated cytotoxic T_{EM} cells, which could play a critical role in the progressive destruction of biliary epithelial cells. (HEPATOLOGY 2011;54:1293-1302)

Primary biliary cirrhosis (PBC) is a female-predominant, organ-specific autoimmune disease characterized by destruction of intrahepatic small bile duct biliary epithelial cells (BECs).[1] The serological hallmark of PBC is the presence of antimitochondrial autoantibodies (AMAs) directed against the pyruvate dehydrogenase E2 complex (PDC-E2) located in the inner membrane of mitochondria.[2-4] High

Abbreviations: AMA, antimitochondrial antibodies; APC, allophycocyanin; BEC, bile duct biliary epithelial cell; BSA, bovine serum albumin; CD, cluster of differentiation; CVH, chronic viral hepatitis; EDTA, ethylenediaminetetraacetic acid; ELC, EBI1-ligand chemokine; ELISA, enzyme-linked immunosorbent assay; FcR, Fc receptor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HLA, human leukocyte antigen; HNK-1, human natural killer-1; HPLC, high-performance liquid chromatography; IFN-γ, interferon gamma; IP-10, IFN-γ-inducible protein 10; IL, interleukin; IgG, immunoglobulin G; LGL, large granular lymphocyte leukemia; mAb, monoclonal antibody; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MFI, mean fluorescent intensity; NASH, nonalcoholic steatohepatitis; NK, natural killer; PBC, primary biliary cirrhosis; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PD-1, programmed death 1; PDC-E2, E2 component of pyruvate dehydrogenase; PE, phycoerythrin; RPMI, Roswell Park Memorial Institute; SEM, standard error of the mean; SLC, secondary lymphoid tissue chemokine; TCR, T-cell receptor; TECK, thymus-expressed chemokine; T_{EM}, effector memory T cell; thymus-expressed chemokine CCL25, CC chemokine ligand 25; TIM-3, T-cell immunoglobulin mucin-3.

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frequency of cluster of differentiation (CD)4^+ and $CD8^+$ T-cell infiltrates have been noted within the portal tracts of the PBC liver, which strongly suggests that these cells are involved in the pathogenesis of PBC.[5] Indeed, PDC-E2-specific autoreactive $CD4^+$ T and $CD8^+$ T cells have been identified both in peripheral blood and, at much higher levels, in the liver of PBC patients.[6-8] The dominant $CD4^+$ and $CD8^+$ T-cell epitopes on PDC-E2 have been mapped.[6-8]

Although both $CD4^+$ and $CD8^+$ T cells are present within portal tract infiltrates, there is a growing body of data that suggests a more direct role of cytotoxic $CD8^+$ T cells in biliary destruction.[9-11] The study of effector pathways is a particularly challenging problem in human autoimmunity. For one thing, the majority of effector pathways are likely to be mediated by nonspecific bystander cells recruited during inflammation. For another, it has been difficult to identify subpopulations of cells by phenotype and thence link such data to functionality. Our laboratory has focused attention on effector T-cell populations, using a variety of technologies, and has highlighted the important role of T cells in this and similar pathways.

In this study, we took advantage of newer reagents, including cell-surface markers, that are associated with $CD8^{high}$ effector memory T cells (T_{EM}), organ- and tissue-specific homing, and alterations in susceptibility to apoptosis. Indeed, we report that patients with PBC not only have an increased frequency of $CD45RO^{high}CD57^+CD8^{high}$ T cells, compared to controls, but also that such cells have increased $\alpha4\beta7$ expression with concurrently increased expression of CCR5 and decreased expression of CCR7 and CD28, compared to other $CD8^{high}$ T cells. Furthermore, this T-cell subset has increased the production of granzyme A, granzyme B, and perforin, compared with other $CD8^{high}$ T cells, and, interestingly, have decreased stimulation-induced apoptosis. Furthermore, interferon gamma (IFN-γ) and interleukin (IL)-5 produced by $CD8^+CD57^+$ T lymphocytes upon *in vitro* T-cell-receptor (TCR) stimulation are increased in PBC patients. Histologically, $CD8^+CD57^+$ T cells accumulate around the portal area in the liver of PBC patients. Moreover, purified $CD8^+CD57^+$ T cells from PBC patients specifically respond to the major histocompatibility class I restricted epitope of PDC-E2. These data have implications for understanding CD8 effector pathways in this autoimmune disease. We submit that $CD45RO^{high}CD57^+CD8^{high}$ T cells are a subset of cytotoxic memory cells, which play a critical role in the chronic, progressive destruction of BECs in PBC.

Materials and Methods

Subjects. Heparinized (Vacutainer; BD Biosciences, Franklin Lakes, NJ) peripheral blood samples were obtained from 76 PBC patients (59.0 ± 1.0 years; mean ± standard error of the mean [SEM]) and 56 age-matched healthy controls (54.8 ± 1.5 years). The diagnosis of PBC was based on internationally accepted criteria.[12] Stage of disease was established according to Ludwig et al..[13] In the present study, 50 of 76 (65.8%) patients with PBC were stage I or II and 22 of 76 (28.9%) were III or IV, whereas 5 of 76 (6.6%) patients were AMA negative (Table 1). We did not observe any difference between AMA-positive and -negative patients; hence, the data are combined herein. The study was approved by the Institutional Review Board of the University of California at Davis (Davis, CA), and all subjects provided written, informed consent prior to enrollment.

Peripheral Blood Mononuclear Cell Isolation. Peripheral blood mononuclear cells (PBMCs) from all subjects were isolated by density gradient using Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) under endotoxin-free conditions. PBMCs were resuspended in phosphate-buffered saline (PBS) (Mediatech Inc., Herndon, VA), containing 0.5% bovine serum albumin (BSA) (Fraction V, OmniPur; EMD Chemicals Inc., Gibbstown, NJ) and 0.05% ethylenediaminetetraacetic acid (EDTA). The viability of cells was >98%, which was confirmed using trypan blue dye exclusion.

Evaluation of Cell Phenotypes. The polychromatic phenotypic analysis of PBMCs was carried out on a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA) upgraded for the detection of five colors by Cytek Development (Fremont, CA). Cells were stained with different combinations of fluorochrome-conjugated monoclonal antibodies (mAbs), including CCR5, CD8b, CCR7, and CD45RO (BD Pharmingen, San Diego, CA), CCR9 (R&D Systems, Minneapolis, MN), CD56, CXCR3, CD57, CD8a, CD45RO, CD28, and CD16 (BioLegend, San Diego,

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Potential conflict of interest: Nothing to report.

Table 1. Clinical Characteristics of PBC Patients*

PBC (n = 76)	AMA Positive	AMA Negative	Age: 59.0 ± 1.0
Stage I	n = 32	n = 1	Early stage (65.8%)
Stage II	n = 15	n = 2	
Stage III	n = 9	n = 1	Late stage (28.9%)
Stage IV	n = 11	n = 1	
Unknown	n = 4		

*There were 56 controls (age, 54.8 ± 1.5 years).
Abbreviations: PBC, primary biliary cirrhosis; AMA, antimitochondrial antibodies.

CA), and CCR7 (eBioscience, San Diego, CA). The allophycocyanin (APC)-conjugated anti-$\alpha 4\beta 7$ was produced in our laboratory. Immunoglobulin G (IgG) isotype controls with matching conjugates for each antibody were used as negative controls. PBMCs were resuspended in staining buffer (0.2% BSA, 0.04% EDTA, and 0.05% sodium azide in PBS), divided into 25-μL aliquots, and incubated with antihuman Fc receptor (FcR) blocking reagent (eBioscience) for 15 minutes at 4°C. Cells were then washed and stained with the antibody cocktails for 30 minutes at 4°C. Cells were washed once with PBS containing 0.2% BSA. For intracellular staining, cells were first stained with phycoerythrin (PE)-anti-CD57 (BioLegend), PerCP-anti-CD8a (BioLegend), APCe780-anti-CCR7 (eBioscience), and APC-anti-CD45RO (BioLegend), then fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Pharmingen) for 15 minutes at 4°C. Subsequently, intracellular staining was performed with AF488-labeled antigranzyme A (BioLegend), AF488-labeled anti-Perforin (BioLegend), or fluorescein isothiocyanate (FITC)-labeled antigranzyme B (BD Pharmingen) or IgG isotype controls. After staining, cells were washed and fixed with 1% paraformaldehyde in PBS. Acquired data were analyzed with Cellquest PRO software (BD Immunocytometry Systems).

Isolation and Culturing of $CD57^+CD8^+$ T Cells. $CD8^+$ T cells were isolated with RosetteSep™ human $CD8^+$ T cell enrichment cocktails (StemCell Technologies, Vancouver, British Columbia, Canada), following the manufacturer's instructions, then resuspended in PBS containing 2% fetal bovine serum (FBS). The $CD57^+$ $CD8^+$ T-cell subset was isolated from the enriched $CD8^+$ T cells using human CD57 MicroBeads (Miltenyi Biotec, Auburn, CA). An aliquot of the isolated $CD57^+$ population was analyzed for purity with flow cytometry, which was always >92%. Aliquots of $CD57^+CD8^+$ T cells (2×10^5) were cultured in 96-well, round-bottomed plates in 200 μL of Roswell Park Memorial Institute (RPMI) medium with 10% heat-inactivated FBS (Gibco-Invitrogen Corp., Grand Island, NY), 100 μg/mL of streptomycin, 100 U/mL of penicillin, and 0.5 μg/mL each of anti-CD3 (BioLegend) and anti-CD28 (BioLegend). Cells were incubated for 5 days at 37°C in a humidified 5% CO_2 incubator, then centrifuged. The supernatant was collected for cytokine analysis.

Cytokine Detection. Supernatant from cultured $CD57^+$ $CD8^+$ T cells was analyzed with enzyme-linked immunosorbent assay (ELISA) kits for IFN-γ (R&D Systems), granzyme A (Bender MedSystems, Vienna, Austria), and IL-5 (BioLegend).

Apoptosis. To assess the relative susceptibility of *in vitro* stimulated $CD45RO^{high}CD57^+CD8^{high}$ T cells to apoptosis, 1×10^6 PBMCs were cultured in 48-well, flat-bottomed plates in 1 mL of RPMI 1640 (Gibco-Invitrogen Corp.), supplemented with 10% heat-inactivated FBS, 100 μg/mL of streptomycin, 100 U/mL of penicillin, 5 μg/mL of anti-CD3 (BioLegend), and 5 μg/mL of anti-CD28 (BioLegend). Cultures were incubated at 37°C in 5% CO_2. After 48 hours of culturing, cells were washed twice with 0.2% BSA in PBS, and the frequency of cells undergoing apoptosis was determined with flow cytometry, using FITC-conjugated anti-Annexin-V (BD Pharmingen), following the manufacturer's instructions. Cells were also stained with Fas (CD95), programmed death 1 (PD-1) (Biolegend), and T-cell immunoglobulin mucin-3 (TIM-3) (eBioscience) after culture.

Synthetic Peptide Assay. PBMC from a nested study, including 3 patients with PBC (PBC 1-3) who were human leukocyte antigen (HLA) A2.1 and 6 other patients with PBC (PBC 4-9) who had other class I alleles, were isolated. As controls, PBMCs from 4 healthy HLA A2.1 controls and 5 HLA A2.1 negative were collected. The peptide, 159-167 of PDC-E2 (KLSEGDLLA), was synthesized by F-moc chemistry (Model Synergy; Applied Biosystems Inc., Foster City, CA). This peptide was purified by reverse-phase high-performance liquid chromatography (HPLC), and the purity was more than 80% as determined by HPLC analysis. Aliquots of $CD57^+CD8^+$ T cells or $CD57^-CD8^+$ T cells (2×10^5) were cultured in the presence of autologous irradiated (3,000 rad) APC (2×10^5), with or without the 159-167 synthetic peptide, for 5 days. The supernatant was collected for cytokine analysis. Controls were used throughout all assays. Supernatant from the cultured cells was analyzed by ELISA for IFN-γ (R&D Systems).

Immunohistochemistry. Liver sections were immunostained using our standard microwave protocol, as previously described.[14,15] All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin, then 4-μm-thick sections were cut from each paraffin

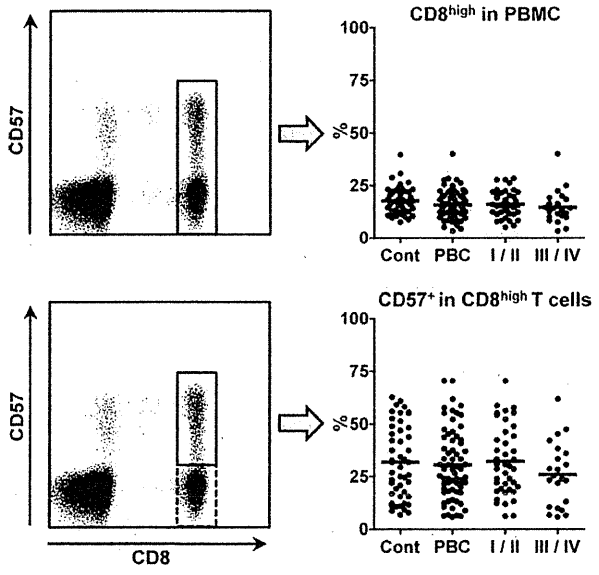

Fig. 1. Analysis of the frequencies of $CD8^{high}CD57^{+}$ cells in lymphocyte population from PBC patients and healthy subjects. Left panels: representative dot plot of gated lymphocyte population. Right panels: percentage of the gated $CD8^{high}$ in PBMC (upper panel) or $CD57^{+}$ in $CD8^{high}$ lymphocytes (lower panel) populations in healthy controls (cont), all PBC patients (PBC), and PBC patients at early stages (I/II) and late stages (III/IV). Bars denote mean percentages.

block from 4 patients with PBC, 3 with chronic viral hepatitis (CVH), and 1 with nonalcoholic steatohepatitis (NASH). The following antibodies were used for the detection of CD8 and CD57 in human liver specimens: rabbit polyclonal antibody against CD57 (Novus Biologicals, Littleton, CO), antihuman CD8 antibody (mAb; DAKO, Glostrup, Denmark) and Envision-peroxidase (DAKO). In all samples, predetermined optimal dilutions were used, and positive and negative samples were included with each assay, and the data were interpreted by a "blinded" pathologist (K.T.).

Statistical Analysis. The percentages of $CD8^{high}$ T-cell subsets that express individual cell markers in PBC patients and healthy controls were expressed as mean ± SEM and compared with the two-tailed Mann-Whitney U test. A P value <0.05 was considered statistically significant. Percentages of the $CD45RO^{high}CD57^{+}CD8^{high}$ T-cell subset and other $CD8^{high}$ T-cell subsets were analyzed using a two-tailed Wilcoxon matched-pairs test.

Results

Increased Frequency of $CD45RO^{high}CD57^{+}CD8^{high}$ T Cells in Peripheral Blood of PBC Patients. There were no significant differences observed in the mean frequencies of $CD8^{high}$ T cells in the PBMC and $CD57^{+}$ cells in $CD8^{high}$ T cells of PBC patients, compared with control subjects (Fig. 1). However, the frequency of $CD45RO^{high}CD57^{+}$ cells were significantly higher in $CD8^{high}$ T cells of PBC patients (7.15% ± 0.77%), in particular patients at earlier disease stages (8.25% ± 1.16%), compared with healthy controls (4.10% ± 0.37%; $P < 0.0005$) (Fig. 2). The $CD45RO^{high}CD57^{+}CD8^{high}$ population did not include natural killer (NK) cells, as the vast majority of these cells were $CD3^{+}$ (99.9% ± 0.14%) and $CD16^{-}$ (98.69% ± 0.69%) (data not shown).

Increased Expression of $\alpha4\beta7^{high}$ and Decreased CD28 on $CD45RO^{high}CD57^{+}CD8^{high}$ T Cells in PBC Patient. To further characterize the $CD45RO^{high}CD57^{+}CD8^{high}$ T-cell subset, these cells were analyzed for their expression of a panel of phenotypic markers, including multiple chemokine receptors, $\alpha4\beta7$ integrin, and the costimulatory molecule, CD28. Results of these studies (Fig. 3) demonstrate that whereas the frequency of the $CD45RO^{high}CD57^{+}CD8^{high}$ subset expressing the gut homing $\alpha4\beta7^{high}$ integrin was significantly higher in PBC patients (18.51% ± 1.94%) than that in controls (11.69% ± 1.41%; $P < 0.03$) and decreased the

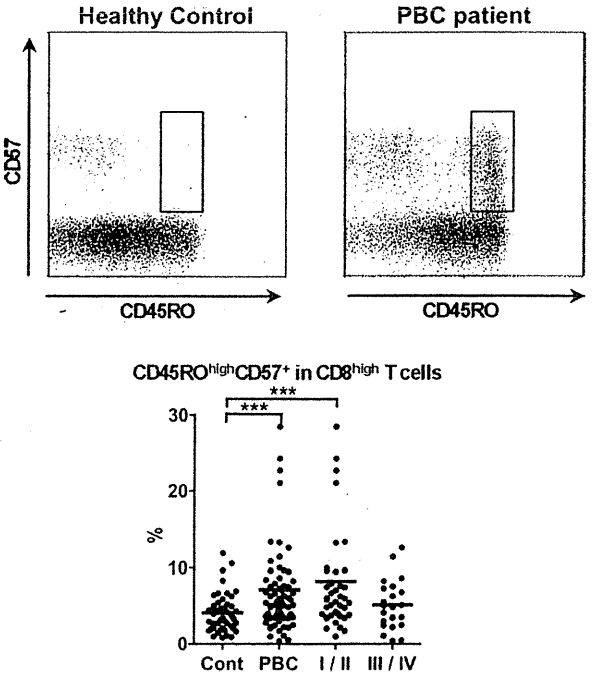

Fig. 2. Analysis of the frequency of the $CD57^{+}$ and $CD45RO^{high}$ cells in $CD8^{high}$ lymphocytes from PBC patients and healthy subjects. Top panels: representative dot plots of $CD8^{high}$ gated lymphocyte population from a healthy control and a patient with PBC. Bottom panel: comparison of the mean percentages of the gated $CD45RO^{high}CD57^{+}$ population in $CD8^{high}$ lymphocytes, among healthy controls (cont), all PBC patients (PBC), and PBC patients at early stages (I/II) and late stages (III/IV). Bars denote mean percentages. $***P < 0.001$.

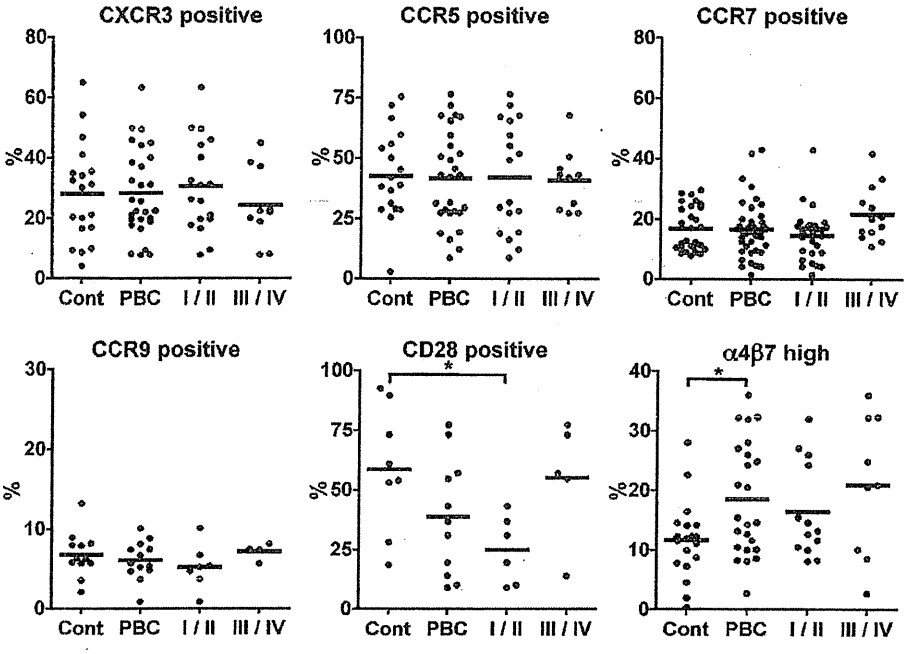

Fig. 3. Analysis of the expression of CXCR3, CCR5, CCR7, CCR9, CD28, and $\alpha 4\beta 7$ on $CD45RO^{high}CD57^{-}CD8^{high}$ gated T lymphocytes from healthy controls (cont), all PBC patients (PBC), and PBC patients at early stages (I/II) and late stages (III/IV). Bars denote the mean percentages. *$P < 0.05$.

expression of CD28 in early-stage PBC patients (25.01% ± 5.81%) than controls (58.76% ± 9.36%; $P < 0.03$), there were no significant differences in the frequencies of the cells expressing the chemokine receptors, CXCR3 (a receptor for IFN-γ-inducible protein 10 [IP-10] and the monokine, Mig), CCR5 (a receptor for RANTES and MIP-1α,β), and CCR7 (a receptor for EBI1-ligand chemokine [ELC] and secondary lymphoid tissue chemokine [SLC]), compared with controls. Of interest, there was no difference in the frequencies of this $CD8^{high}$ subset that expressed the gut-homing chemokine receptor, CCR9, a receptor for thymus-expressed chemokine (TECK)/CC chemokine ligand 25 (thymus-expressed chemokine CCL25).

Altered Granzyme and Perforin Expression in $CD45RO^{high}CD57^{+}CD8^{high}$ T Cells. In PBC patients, we have compared phenotypes of $CD45RO^{high}CD57^{+}CD8^{high}$ T cells to other $CD8^{high}$ T cells. In addition to the analysis of homing and chemokine receptors, we also studied the cytotoxic potential of the $CD45RO^{high}CD57^{+}CD8^{high}$ subset of T cells. Interestingly, though there was a significant increased expression of CCR5 and $\alpha 4\beta 7^{high}$, and significantly decreased expression of CCR7 and CD28 in $CD45RO^{high}CD57^{+}CD8^{high}$ T cells, compared to other $CD8^{high}$ T cells in PBC patients, there was no difference observed in $\alpha 4\beta 7^{high}$ and CD28 expressions in healthy controls (Fig. 4A,C). CCR5, CCR7, granzyme A, granzyme B, and perforin demonstrated a similar phenotypic pattern in PBC and healthy controls (data not shown). In addition, relative to other $CD8^{high}$ T cells, $CD45RO^{high}CD57^{+}CD8^{high}$ T cells had increased production of granzyme A ($P < 0.001$), granzyme B ($P < 0.001$), and perforin ($P < 0.001$), suggesting their strong cytotoxic effector functions (Fig. 4B).

The $CD45RO^{high}CD57^{+}CD8^{high}$ T Cells Are Not Susceptible to Apoptosis Upon Anti-CD3 Stimulation. $CD57^{+}$ T cells have previously been demonstrated to be susceptible to apoptosis during chronic antigenic stimulation.[16] Therefore, we determined the relative susceptibility of $CD45RO^{high}CD57^{+}CD8^{high}$ T cells from PBC patients to undergo apoptosis. PBMCs from PBC patients and controls were stimulated by anti-CD3/28 for 48 hours, then examined for Annexin V expression by the $CD45RO^{high}CD57^{+}CD8^{high}$ T cells. Interestingly, Annexin V expression was decreased in $CD45RO^{high}CD57^{+}CD8^{high}$ T cells from PBC patients (35.23% ± 3.07%), specifically those with early disease stages (32.68% ± 4.02%), compared with healthy controls (46.18% ± 1.51%; $P < 0.03$) (Fig. 5A). We further investigated the expression of Fas, TIM-3, and PD-1, and found that PD-1 expression was significantly decreased in PBC patients (51.8% ± 6.54%), compared to that in healthy controls (75.03% ± 7.12%; $P < 0.02$) (Fig. 5A). This reduced susceptibility to stimulation-induced apoptosis was not observed in other $CD8^{high}$ T cells, suggesting a unique apoptosis resistance in the $CD45RO^{high}CD57^{+}CD8^{high}$ T cells from PBC patients.

The $CD57^{+}CD8^{+}$ T-Cell Subset From PBC Patients Produce Increased Levels of IL-5, IFN-γ, and Granzyme A. To investigate the cytokine profile of $CD57^{+}CD8^{+}$ T cells, $CD57^{+}CD8^{+}$ T cells

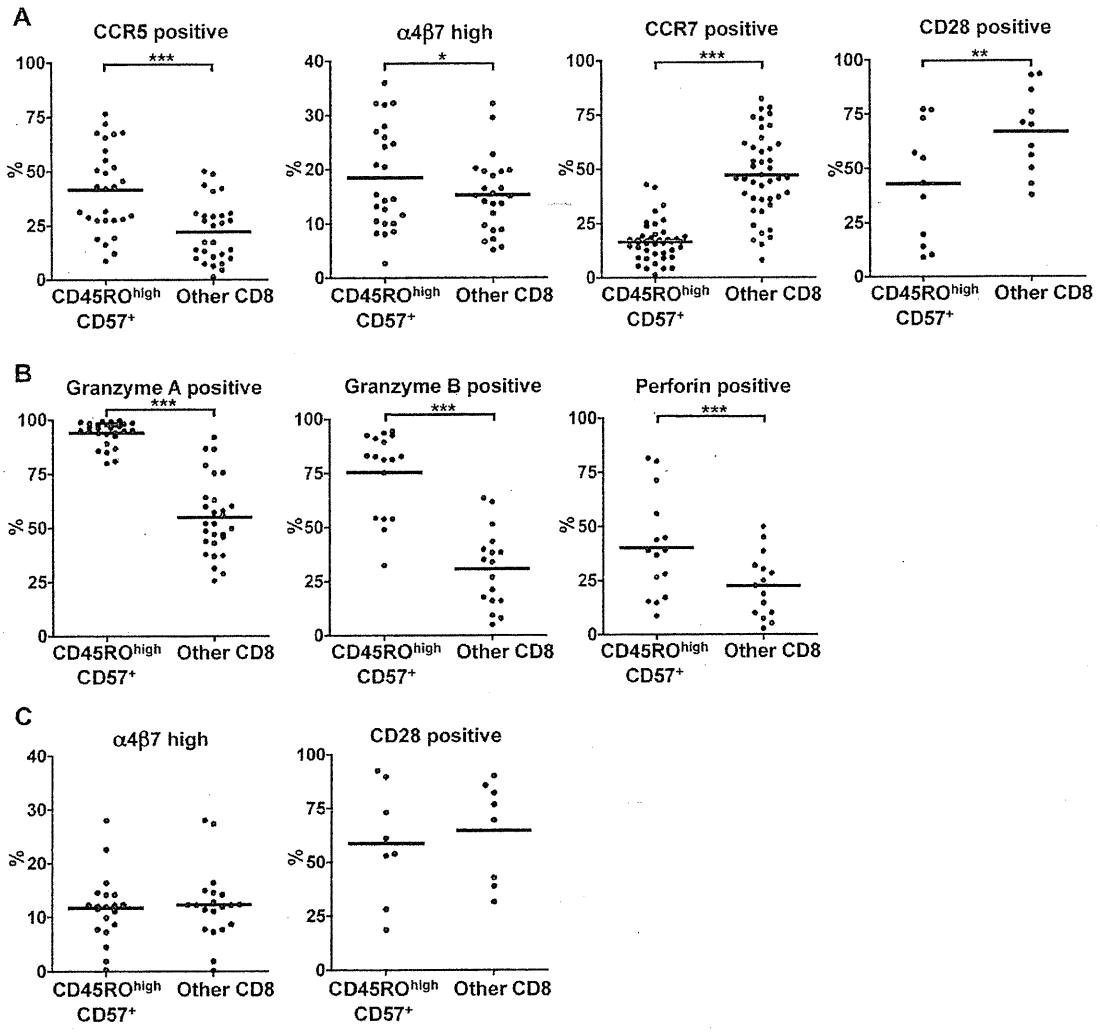

Fig. 4. Comparison of phenotypes between $CD45RO^{high}CD57^{+}CD8^{high}$ T cells and other $CD8^{high}$ T cells. The other $CD8^{high}$ T cells include $CD45RO^{-\text{ to }low}CD57^{+}CD8^{high}$ and $CD57^{-}CD8^{high}$ cells. Bars denote mean percentages. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (A) Cell-surface markers in PBC. (B) Intracellular markers in PBC. (C) Cell-surface markers in healthy control.

isolated from PBC patients and healthy controls were stimulated *in vitro* with anti-CD3/28 for 5 days. Supernatants were collected and analyzed for levels of secreted IFN-γ, IL-5, and granzyme A. As shown in Fig. 6, $CD57^{+}CD8^{+}$ T cells from PBC patients secreted increased levels of IL-5 (157.4 ± 47.7 pg/mL), compared with controls (30.0 ± 7.7 pg/mL; $P < 0.001$). No significant difference was observed in the levels of IFN-γ and granzyme A.

$CD8^{+}CD57^{+}$ Cells Were Infiltrated in Hepatic Portal Track and Respond to the HLA-A2-Restricted CTL Epitope, PDC-E2. $CD57^{+}$cells coexisted in the area of $CD8^{+}$ infiltration (i.e., were $CD8^{+}CD57^{+}$ double positive). In contrast, it was uncommon to have $CD57^{+}$ coexpression detected in $CD8^{+}$-infiltrating control livers (Fig. 7A). To further assess the prevalence of autoreactive T cells, we investigated the difference in response with the HLA-A2-restricted cytotoxic T lymphocyte (CTL) epitope. $CD8^{+}CD57^{+}$cells from HLA-A2.1-positive PBC patients (124.3 ± 16.5 pg/ml) had increased production of IFN-γ upon PDC-E2 stimulation, compared to $CD8^{+}CD57^{+}$cells from HLA-A2.1-positive healthy controls (39.7 ± 10.1 pg/ml); as expected, there was no significant difference between PBC non-HLA-A2.1 patients versus controls (Fig. 7B).

Discussion

In this study, we have carried out a comprehensive phenotypic and functional characterization of $CD8^{+}$ T cells, which is believed to be directly responsible for the destruction of BECs in PBC. Our results

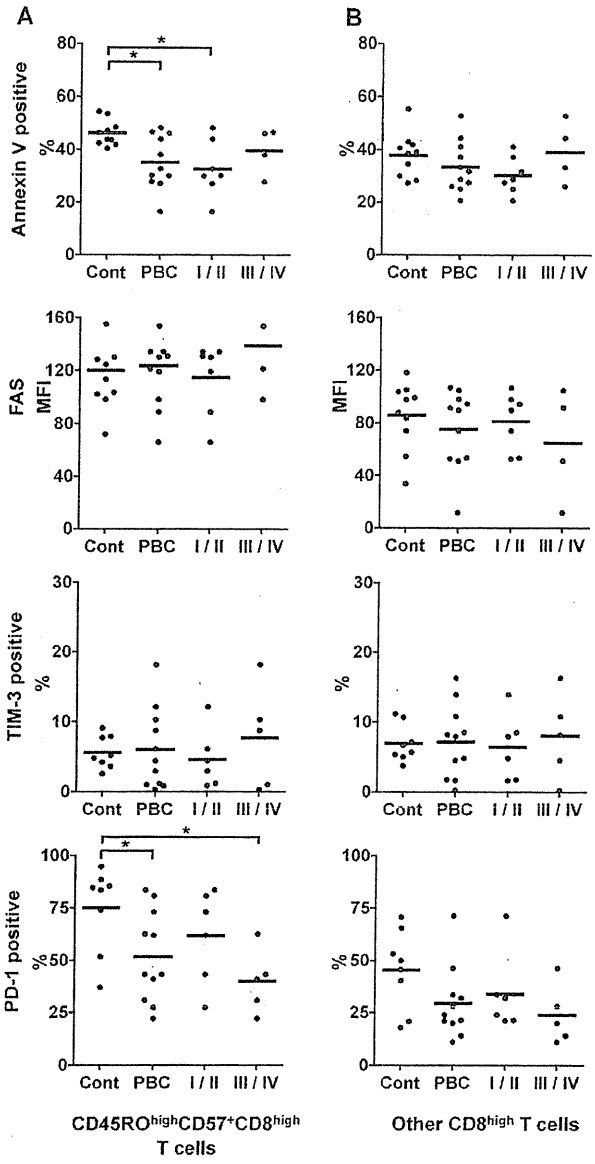

Fig. 5. Comparison of the expression of Annexin V, Fas, TIM-3, and PD-1 on $CD45RO^{high}CD57^{+}CD8^{high}$ T cells and other $CD8^{high}$ T lymphocytes between PBC patients and healthy subjects. PBMCs were stimulated with anti-CD3/28, then stained for Annexin V, Fas, TIM-3, and PD-1. (A) Percentage of Annexin V, TIM-3, and PD-1-positive cells or mean fluorescent intensity (MFI) of Fas in $CD45RO^{high}CD57^{+}CD8^{high}$ T cells from healthy controls (cont), all PBC patients (PBC), and PBC patients at early stages (I/II) and late stages (III/IV). (B) Percentage of Annexin V, TIM-3, and PD-1 positive cells or MFI of Fas in other $CD8^{high}$ T cells. See Fig. 4 legend for the definition of other $CD8^{high}$ T-cell populations. Bars denote mean percentages. $*P < 0.05$.

demonstrate the following: (1) PBC patients had an increased frequency of $CD45RO^{high}CD57^{+}$ cells in $CD8^{high}$ T cells, compared with age-matched healthy controls; (2) $CD45RO^{high}CD57^{+}CD8^{high}$ T cells from PBC patients more frequently expressed $\alpha4\beta7$ and demonstrated reduced CD28 expression, compared with controls; (3) in PBC patients, the $CD45RO^{high}CD57^{+}CD8^{high}$ subset had increased frequency of $CCR5^{+}$ and $\alpha4\beta7^{high}$ cells, decreased frequency of $CCR7^{+}$ and $CD28^{+}$ cells, and expressed increased levels of granzyme A, B, and perforin, in comparison to other $CD8^{high}$ T cells, consistent with an effector memory phenotype; (4) upon CD3 stimulation, $CD57^{+}CD8^{+}$ T cells from PBC patients were less prone to apoptosis while having secreted increased levels of IL-5 than healthy controls; and (5) $CD57^{+}CD8^{+}$ T cells infiltrate the PBC liver portal area; this cell population demonstrates autoreactivity against the HLA-A2.1, the restricted epitope.

It is of interest to note that the $CD57^{+}CD8^{+}$ T-cell subset has been previously described as possessing both cytotoxic and regulatory functions.[17-21] In our present study, the results suggest that a subpopulation of the $CD57^{+}CD8^{+}$ T cells, namely $CD45RO^{high}CD57^{+}CD8^{high}$ T cells, is a subset of cytotoxic effector memory cells that could be critical in cell-mediated immune response in PBC. The CD57 antigen is a glycoepitope that was first described on human NK 1 (HNK-1) cells.[22] An increase in the frequency of $CD57^{+}$ T cells has been reported in patients after bone marrow and solid organ transplants,[23,24] in rheumatoid arthritis,[25,26] and acquired immune deficiency patients.[27] These studies have suggested a role for such $CD57^{+}$ T cells in the immunological abnormalities manifested in such diseases. Although the frequency of the $CD8^{+}CD57^{+}$ T cells in normal hosts ranges from 5% to 20%,[28] the frequency of this subset increases with aging.[29,30] Although several studies have suggested an augmented cytotoxic ability of the $CD8^{+}CD57^{+}$ T cell,[19-21] there is a paucity of data on this $CD8^{+}CD57^{+}$ T-cell population in PBC.

The present results provide further insights into the potential mechanisms by which $CD8^{+}$ cytotoxic T cells serve as effector cells in the pathogenesis of PBC.[7,9,11] We demonstrate herein that the $CD45RO^{high}$ subset of $CD57^{+}CD8^{high}$ cells were more resistant to stimulation-induced apoptosis, as compared to their counterparts, in the control subjects, which is similar to the finding that the $CD57^{+}CD8^{+}$ T-cell population in PBMCs from patients with large granular lymphocyte leukemia (LGL) were resistant to Fas-stimulated apoptosis (31).[31] This resistance to apoptosis is demonstrated by lower expression of Annexin V and PD-1. PD-1/PD-L interaction plays a critical role in $CD8^{+}$ T-cell tolerance[32]; previous work has demonstrated that a decrease in PD-1 signaling can generate murine autoimmune hepatitis.[33]

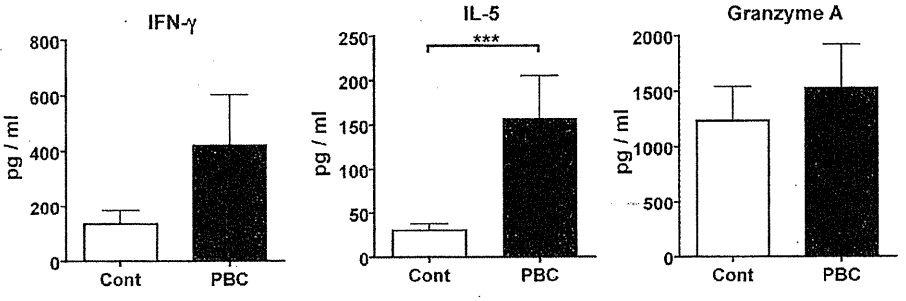

Fig. 6. Production of IFN-γ, IL-5, and granzyme A by $CD57^{+}CD8^{+}$ T cells from PBC patients and healthy controls. Aliquots of isolated $CD57^{+}CD8^{+}$ T cells from PBC patients (n = 7) and healthy controls (n = 8) were cultured in duplicates in the presence of anti-CD3/28 (0.5 μg/mL) for 5 days. $^{***}P < 0.001$.

We reasoned that examination of chemokine receptors and the integrin, α4β7, which provide homing signals for circulating leukocytes to migrate to disease-specific tissues, would provide evidence that these circulating cells reflect the immune response in the target organ.[34] T-cell recruitment to the liver is orchestrated by a series of adhesion molecules and homing chemokines.[35] We demonstrate herein that the $CD45RO^{high}$ subset of $CD57^{+}CD8^{high}$ cells more frequently expressed the homing integrin, α4β7. Although the integrin, α4β7, and chemokine receptor, CCR9, are typically associated with gut-homing phenotypes, they have also been shown to mediate the adhesion of liver-infiltrating lymphocytes through the expression of their cognate ligands. Specifically, hepatic expression of the α4β7 ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), has been demonstrated in a variety of liver diseases, including PBC.[35] The CCR9 ligand and the presumed gut-specific chemokine, CCL25, is expressed primarily by epithelial cells that line the small intestine and has also been shown to be expressed on hepatic endothelial cells of patients with primary sclerosing cholangitis, but, in contrast to MAdCAM-1, not in PBC.[36] Thus, it is not surprising that, in our study, there was an increased frequency of $CD45RO^{high}CD57^{+}CD8^{high}$ T cells expressing α4β7, but not CCR9.

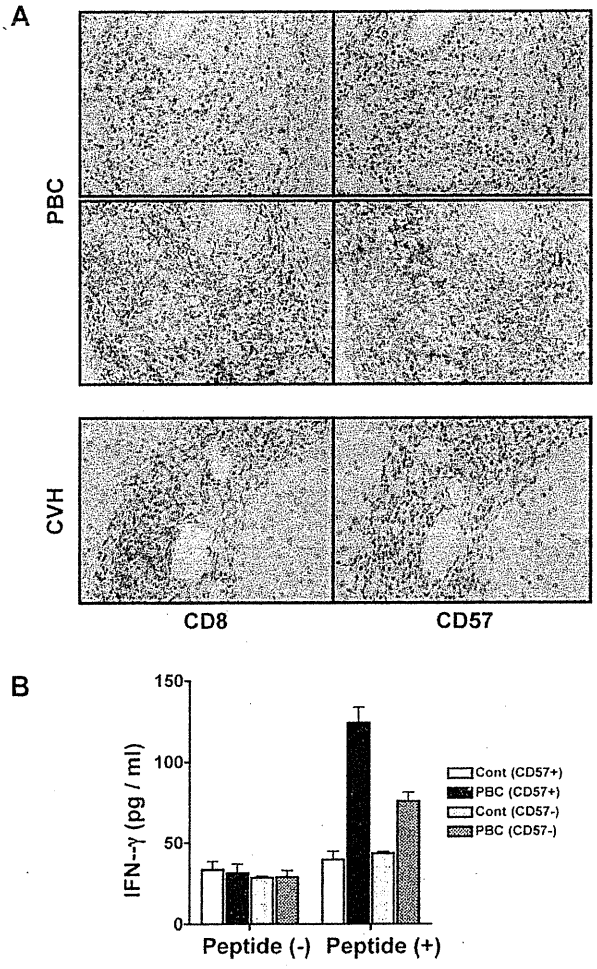

Fig. 7. (A) Liver immunohistochemistry from representative PBC and CVH patients. Top and middle rows demonstrate 100% near complete (top row) to 70% (middle row) CD57-positive staining within the CD8-positive area in PBC; only minor CD57-positive staining was detected within the CD8-positive area in CVH, as shown in the bottom row. (B) IFN-γ production upon PDC-E2 peptide stimulation. Data from HLA-A2.1-positive PBC patients (n = 3) and healthy controls (n = 4) are shown. Left bar graphs are without peptide stimulation, and right bar graphs are with peptide stimulation.

In a murine model of graft-versus-host disease, which develops both portal hepatitis and nonsuppurative destructive cholangitis similar to PBC, CCR5-expressing $CD8^{+}$ T cells migrate into the portal areas of the liver and play a significant role in causing liver injury.[37] The increased expression of CCR5 by $CD45RO^{high}CD57^{+}CD8^{high}$ T cells, but not other $CD8^{high}$ T cells, suggests that $CD45RO^{high}CD57^{+}CD8^{high}$ T cells play an active role as effector cells during bile duct destruction in PBC. The expression of CD28 decreases when $CD8^{+}$ T cells differentiate from memory to effector $CD8^{+}$ T cells.[38] The decrease in CD28 expression observed in PBC, especially in early-stage PBC patients, implicates strong effector function with autoreactive properties of $CD45RO^{high}CD57^{+}CD8^{high}$ T cells.

Also, our observation of decreased CCR7 expression on $CD45RO^{high}CD57^{+}CD8^{high}$ T cells, compared with other $CD8^{high}$ T cells, is consistent with the

theory that these cells are effector memory cells, as opposed to $CCR7^+$ central memory cells, which express lymph-node homing receptors and lack immediate effector function.[39] It is reasoned that the lymph-node homing $CD8^{high}$ T cells may become mobilized to the periphery and acquire a different spectrum of cell-surface molecules while decreasing the levels of CCR7 expression during this process.

Our data demonstrate an increased secretion of IL-5 by $CD57^+CD8^+$ T cells, compared with a similar population from controls. Increased IL-5 has also been found in other studies of $CD8^+CD57^+$ T lymphocytes.[40] The transcripts for both Th1- and Th2-type cytokines, such as INF-γ, IL-2, and IL-5, are upregulated in the blood and liver of PBC,[41,42] and IL-5 promotes the differentiation of activated B cells into Ig-producing cells and augments both IgM and IgA production.[43,44] Moreover, IL-5 has potent, specific effects on eosinophil activation and degranulation.[45,46] Eosinophilia has been demonstrated in PBC patients, and eosinophil cytotoxic products, such as major basic protein, have been localized to the periportal regions of the patient liver.[42,47] Our data demonstrate that $CD57^+CD8^+$ T cells are a potential source of IL-5 during the chronic stages of PBC, exacerbating the destruction of BECs. $CD57^+$ $CD8^+$, in particular $CD45RO^{high}CD57^+CD8^{high}$, T cells may also contribute to continuous AMA production in PBC.

Collectively, our data demonstrate that $CD57^+$ $CD8^{high}$ T cells are a subset of cytotoxic memory T cells that include specific autoreactive $CD8^+$ T cells. Our results demonstrate, for the first time, the increased frequency of $CD45RO^{high}CD57^+CD8^{high}$ T cells with the unique increased expression of $\alpha 4\beta 7$ integrin and CCR5 as well as resistance to apoptosis in PBC PBMCs; this reflects a role of $CD45RO^{high}CD57^+CD8^{high}$ T cells as a $CD8^+$ subpopulation contributing to the progressive destruction of small bile ducts. We do not imply that the data herein will be unique only to patients with PBC and, indeed, may well be a property of multiple other autoimmune diseases, obviously with different antigenic specificity and tissue-specific homing receptors. We do suggest, however, that further studies focused on these effector mechanisms will enable the dissection of the role of $CD8^+$ subpopulations in PBC.

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Interaction Between Toll-Like Receptors and Natural Killer Cells in the Destruction of Bile Ducts in Primary Biliary Cirrhosis

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Primary biliary cirrhosis (PBC) is characterized by chronic nonsuppurative destructive cholangitis (CNSDC) associated with destruction of small bile ducts. Although there have been significant advances in the dissection of the adaptive immune response against the mitochondrial autoantigens, there are increasing data that suggest a contribution of innate immune mechanisms in inducing chronic biliary pathology. We have taken advantage of our ability to isolate subpopulations of liver mononuclear cells (LMC) and examined herein the role of Toll-like receptors (TLRs), their ligands, and natural killer (NK) cells in modulating cytotoxic activity against biliary epithelial cells (BECs). In particular, we demonstrate that Toll-like receptor 4 ligand (TLR4-L)-stimulated NK cells destroy autologous BECs in the presence of interferon alpha (IFN-α) synthesized by TLR 3 ligand (TLR3-L)-stimulated monocytes (Mo). Indeed, IFN-α production by hepatic Mo is significantly increased in patients with PBC compared to disease controls. There were also marked increases in the cytotoxic activity of hepatic NK cells from PBC patients compared to NK cells from controls but only when the NK cells were prepared following ligation of both TLR3-L- and TLR4-L-stimulated LMC. These functional data are supported by the immunohistochemical observation of an increased presence of CD56-positive NK cells scattered around destroyed small bile ducts more frequently in liver tissues from PBC patients than controls. *Conclusion:* These data highlight critical differences in the varied roles of Mo and NK cells following TLR3-L and TLR4-L stimulation. (HEPATOLOGY 2011;53:1270-1281)

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The cholangitis of primary biliary cirrhosis (PBC) has been called an orchestrated immune attack, including involvement of autoantibodies, $CD4^+$, and $CD8^+$ T cells.[1,2] This concept has led to the thesis that a multilineage response against the immunodominant autoantigen PDC-E2 is an essential component of disease pathogenesis.[3] It is unclear whether the natural history of PBC is "entirely" secondary to adaptive autoimmune responses; epidemiologic analysis has suggested a role of transient exposure

Abbreviations: BEC, biliary epithelial cells; CNSDC, chronic nonsuppurative destructive cholangitis; IFN, interferon; LMN, liver mononuclear cells; mAb, monoclonal antibody; mDC, myeloid dendritic cells; Mo, monocytes; NK cells, natural killer cells; NKT cells, natural killer T cells; PBC, primary biliary cirrhosis; pDC, plasmacytoid dendritic cells; PSC, primary sclerosing cholangitis; TLR, Toll-like receptor; TLR-L, Toll-like receptor ligand; TRAIL, TNF-related apoptosis inducing ligand.

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to environmental agents in the etiology of PBC.[4] The data presented herein suggest that innate immune mechanisms contribute to the pathology characteristic of PBC by either accelerating disease or by specific chronic destruction of small bile duct epithelial cells.[5] Indeed, one paradox in PBC has been the relative lack of a therapeutic response to the various immunosuppressive drugs that have been administered to PBC patients, despite the observation that PBC is a model autoimmune disease.[6] A more detailed analysis of the effector mechanisms involved in the pathogenesis of human PBC has led us to suggest that in addition to the documented adaptive autoimmune responses there is also a direct role of innate immune responses in the biliary pathology of PBC.[2,5,7-9]

The studies described herein take advantage of our ability to culture primary human biliary epithelial cells (BEC) *in vitro* as well as to isolate subpopulations of liver infiltrating mononuclear cells.[8,10,11] Although there are significant numbers of natural killer (NK) cells present around small bile ducts, especially during the early stages of PBC,[12] we note that there are NK cells present throughout the disease course. Importantly, we focused on these NK cells and report herein that such NK cells are highly cytotoxic for autologous BEC following ligation of the Toll-like receptor 4 (TLR4) expressed by NK cells in the presence of interferon-α (IFN-α). Furthermore, this function of NK cells is dependent on the activation of monocytes (Mo) by way of TLR3. We submit that activation of Mo and their crosstalk with NK cells contribute to the pathology of PBC. The data supporting this view are the basis of the present report.

Patients and Methods

Subjects and Protocol. A total of 22 explanted liver tissues constitute the present study. Eight of these 22 liver tissues were from patients with PBC, three from patients with hepatitis B virus infection, eight with hepatitis C virus infection, and three with alcoholic liver disease. The term control diseases in this report refers to patients with diseases other than PBC. All patients had endstage liver cirrhosis without detectable signs of other acute liver injury from an unrelated cause. The diagnosis of PBC was based on established criteria[2] and sera from each of these patients had readily detectable high titers of antimitochondrial antibodies.[2] The immunohistochemical studies reported herein were performed on fresh tissue samples from wedge biopsies of 47 patients including 11 normal controls with metastatic liver disease, 14 patients with PBC, 16 with hepatitis C, and six with primary sclerosing cholangitis (PSC). All of the tissues from patients used herein for immunohistological studies were classified as early stage without detectable signs of cirrhosis. Samples were obtained and studied after informed consent of the donor and all experimental protocols were approved by the Research Ethics Committee of Kyushu University and the University of California at Davis. The isolation, verification of purity, and the specific protocols used are described below.

Isolation of Intrahepatic BECs and Liver-Infiltrating Mononuclear Cells (LMCs). The liver mononuclear cell populations were isolated as described in detail by our laboratory.[7] Briefly, liver specimens were first digested with 1 mg/mL of collagenase type I. Cells from the digested tissue were purified using a Ficoll-hypaque gradient to obtain LMC.[9] The LMC were allowed to adhere by incubating the cells overnight in tissue culture plates and an enriched population of adherent cells harvested. This adherent cell population was maintained in tissue culture until the cells reached full confluence, usually by day 14, and the nonadherent cell population aspirated, washed, and cryopreserved in media containing 7.5% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

BECs were separated from adherent cells using CD326 (EpCAM) conjugated MicroBeads (Miltenyi Biotec) specific for epithelial cells. Cells were then resuspended in media consisting of a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum (FCS), epithelial growth factor (10 ng/mL), cholera toxin (10 ng/mL), hydrocortisone (0.4 μg/mL), triiodothyronine (1.3 μg/L), transferrin (5 μg/mL), insulin (5 μg/mL), adenine (24.3 μg/mL), and 10 ng/mL hepatocyte growth factor (R&D systems, Minneapolis, MN) and cultured.[7] The purity of the cells was verified by immunohistochemical examination of an aliquot of these cells for the expression of cytokeratins 7 and 19 using appropriate antibodies (Dako, Glostrup, Denmark) and only cultures that were >90% positive for these cytokeratins and >95% viable (as determined by trypan blue) were used for the studies reported herein. The cultures used in the studies herein were between four to six passages to exclude the possibility for potential loss of phenotype after prolonged *in vitro* culture.

Isolation of T Cells, Mo, NK Cells, Myeloid Dendritic Cells (mDC), Plasmacytoid DC (pDC), and Natural Killer T (NKT) Cells. As reported,[8] the T cells used for the studies were isolated from LMC using a Pan T cell isolation kit II (Miltenyi Biotec).[8]

Similarly the highly enriched population of Mo and NK cells used were purified using Mo and NK cell isolation kits, respectively (Miltenyi Biotec).[8] The purity of the CD3+ T cells, Mo, and NK cells used were >90% as determined by flow cytometric analysis of an aliquot from each isolation. In efforts to ensure the purity of the cell population being studied, the population of T cells, Mo, or NK cells were each harvested separately. In addition, the same assay was performed following depletion of each of the three cell lineages from LMCs in efforts to confirm that the data obtained were indeed the function of the lineage being studied. The mDCs (BDCA-1+), pDC (BDCA-2+), and NKT cells were isolated using the mDC, pDC, and NKT cell isolation kits (Miltenyi Biotec), respectively, which included two magnetic separation steps. The purity of BDCA-1+ mDCs and the CD3+ CD56+ NKT cells were each >80% as determined by flow cytometric analysis of an aliquot of the cell preparation used for the study. An enriched population of mDC and NKT cells were harvested separately and, once again, the same assay was performed following depletion of the specific cell population in efforts to confirm that the function identified was due to the specific cell lineage being studied.

Cytotoxicity Assay Against Autologous BEC. The cytotoxic activity of LMC was assessed using an 8-hour ^{51}Cr release assay using autologous BEC as target cells.[7] Briefly, the detached BECs were labeled with 2 μCi/mL ^{51}Cr (Amersham) overnight, washed 3× in media and 5 × 10^3 ^{51}Cr-labeled cells dispensed into individual wells of a 96-well round-bottom plate. The nonstimulated, the interleukin (IL)-2, or TLR-activated LMCs were added to triplicate wells at an effector to target cell ratio of 20:1 in a total volume of 200 μL of complete RPMI medium. The IL-2-stimulated effector LMCs used for the assay were stimulated for 3 days with IL-2 (100 units/mL) and the TLR-activated LMC comprised of a series of cell cultures incubated with a single or mixture of TLR ligands each at a predetermined optimal concentration of 2-10 μg/mL of the appropriate TLR-L prior to their addition to the target cells. The TLR ligands used included TLR2 ligand (lipoteichoic acid, LTA: TLR2-L), TLR3 ligand (polyinosine-polycytidylic acid, poly (I:C): TLR3-L), TLR4 ligand (lipopolysaccharide, LPS: TLR4-L), TLR5 ligand (Flagellin: TLR5-L), TLR7/8 ligand (CL097: TLR7/8-L), TLR9 ligand type A (ODN2216, CpG type A: TLR9-LA), and TLR9 ligand type B(ODN2006, CpG type B: TLR9-LB). The combination of TLR ligands used for activation of LMC included (1) TLR2-L + the ligands for either TLR3, 4, 5, 7/8, 9-LA, or 9-LB; (2) TLR3-L + the ligands for either TLR4, 5, 7/8, 9-LA, or 9-LB; (3) TLR4-L + ligands for either TLR5, 7/8, 9-LA, or 9-LB; (4) the TLR5-L + the ligands for either 7/8, 9-LA, or 9-LB; (5) TLR7/8-L + the ligands of either 9-LA to TLR9-LB; (6) TLR9-LA + TLR9-LB. The TLR ligands were purchased from Invitrogen (San Diego, CA). Controls consisted of triplicate wells containing target cells cultured in media alone and target cells that were incubated with 10% Triton X-100 to determine spontaneous and maximal ^{51}Cr release, respectively. Following incubation of the cocultures of the effector with target cells for 8 hours, 100 μL of supernatant fluid was collected from each well and counted and the percentage of specific ^{51}Cr release calculated as (cpm of experimental release − cpm of spontaneous release) / (cpm of maximal release − cpm of spontaneous release) × 100). Experiments using the combination of TLR3-L and TLR4-L were performed on aliquots of samples at least three times from each of the patients. As further controls, polymyxin B and chloroquine were used as specific inhibitors of LPS and poly I:C, respectively, for assays involving TLR4 and TLR3-induced activation. Although polymyxin B was added at the time of TLR4 activation, chloroquine was added 2 hours prior to the activation of the TLR3 pathway for the cytotoxicity assay.

Hepatic Mo, T cells, and NK cells were isolated from LMC following in vitro activation with TLR3-L and TLR4-L for 3 days. Subsequently, highly enriched populations of Mo, T cells, NK cells, and LMC depleted of Mo, T cells, and NK cells were assessed for their cytotoxic activity against autologous BEC at an effector-to-target cell ratio of 5:1. Thence enriched populations of NK cells and LMC were stimulated with several combinations of TLR3-L and TLR4-L in the presence of a variety of supernatant fluids prepared as described above. The combinations included (1) activation of the appropriate cell cultures with TLR3-L and TLR4-L in the presence of supernatant of unfractionated LMC; (2) the activation of the appropriate cell cultures with TLR3-L in the presence of supernatant of TLR4-L-activated LMC; (3) activation of the appropriate cell cultures with TLR4-L in the presence of supernatant of TLR3-L-activated LMC; and (4) activation of the appropriate cell cultures with supernatants of TLR3-L and TLR4-L-stimulated LMC. The stimulated NK and LMC were then assessed for cytotoxicity against autologous BEC. Finally, unfractionated LMC and highly enriched populations of mDC, Mo, NKT, or LMC depleted of mDC, Mo. or NKT cells were cultured at 1 × 10^5/200 μL in 96-well plates for 48 hours in the presence of either TLR3-L or supernatant fluids obtained from cultures of NK

Table 1. Primer Sequences Used for Real-Time Polymerase Chain Reaction Analyses

	PCR Product Size	Forward Primer	Reverse Primer
NKG 2D	200	GTCTCAAAATGCCAGCCTTC	TCGAGGCATAGAGTGCACAG
NKp46	258	ATGGGGCTGTTGAATACCAG	TCTCTCCCGAGATCACTTCG
CD94	279	CCACGGAGTAACATCCCATC	GAAGCTGCAGTGAACCATGA
NKG2A	174	TCCATGGGTGACAATGAATG	CTGCAAATGCAAACGCTTTA
FasL	257	TCTACCAGCCAGATGCACAC	CAAGATTGACCCCGGAAGTA
TRAIL	143	GGCAACTCCGTCAGCTCGTTA	GGTCCCAGTTATGTGAGCTGCTA
Granzyme B	257	TCCCTGTGAAAAGACCCATC	TTCGCACTTTCGATCTTCCT

cells stimulated with TLR4-L. The cultures were then assessed for cytotoxicity against autologous BEC.

In efforts to study the influence of IFN-α, an additional cytotoxicity assay was performed in which highly enriched populations of NK cells were stimulated with TLR4-L in the presence or absence of recombinant IFN-α. In parallel, the supernatant fluids from TLR3-L-stimulated Mo in the presence or absence of anti-IFN-α antibody (Abcam) were studied. Similarly, in nested experiments, anti-TNF-related apoptosis inducing ligand (TRAIL) monoclonal antibody (mAb) (R&D Systems, final concentration: 1 μg/mL), anti Fas-L mAb (R&D Systems, final concentration: 1 μg/mL), or Granzyme B inhibitor (BioVision, final concentration: 10 μM) were used in the same cytotoxicity assay in attempts to identify the effector molecules involved. Importantly, each of these experiments was performed on samples from all PBC patients and control liver disease patients at least three times.

IL-12, IL-15, IL-18, and IFN-α Production from Mo. In efforts to identify the nature of the cytokines that were involved in promoting NK cell effector function, supernatants from the TLR3-L-stimulated hepatic Mo cultured for 3 days were analyzed for levels of IL-12, IL-15, IL-18, and IFN-α. These cytokines were selected based on previously published data that reported their involvement in NK cell functional activity.[13] Assays were performed using a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems), using a combination of unlabeled and biotin- or enzyme-coupled monoclonal antibody to each cytokine. Data reported herein represent results obtained from each of the experiments performed on samples from all patients at least three times.

Isolation and Quantitation of Messenger RNA (mRNA) for Select Markers. Aliquots of NK cells from PBC patients and disease controls were cultured in media alone (unstimulated) or cultured in the presence of TLR4-L, IFN-α, or the combination of TLR4-L and IFN-α for 24 hours. Total RNA was isolated from the cultured NK cells using RNAeasy columns (Qiagen, Valencia, CA) and quantitative analyses carried out utilizing a real-time polymerase chain reaction (PCR) assay using SYBR Green PCR Master Mix (Invitrogen) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Tokyo, Japan). The relative levels of NKG2D and NKp46 (activating receptors), CD94 and NKG2A (inhibitory receptors), and FasL, TRAIL, and Granzyme B (effector function markers) were determined using the primers noted in Table 1. Data are expressed as the fold-change in levels of mRNA versus unstimulated NK cells.

Immunohistochemical Staining of Human Liver Specimens for CD56 Expression. Deparaffinized and rehydrated sections and frozen sections of liver tissues from 11 normal controls with a diagnosis of metastatic liver disease, 14 patients with PBC, 16 with hepatitis C, and six with PSC were used for the detection of CD56-expressing cells using standard immunostaining. Endogenous peroxidase was blocked using normal goat serum diluted 1:10 (Vector Laboratories, Burlingame, CA) for 20 minutes; CD56 was diluted 1:100 (Dako) and immunostaining was performed on coded sections and the data interpreted by a "blinded" pathologist.

Statistical Analysis. All experiments were performed in triplicate and data points shown are the mean values of results of these triplicates. Comparisons between the points for certain datasets are expressed as mean ± standard deviation (SD), and the significance of differences was determined by Student's *t* test. All analyses were two-tailed and *P*-values <0.05 were considered significant. Statistical analyses were performed using Intercooled Stata 8.0 (StataCorp, College Station, TX).

Results

Autologous BEC Killing Assay by LMC. As noted in Fig. 1A and as expected, LMC when cocultured with autologous BEC demonstrated no detectable cytotoxicity (0.5 ± 4.3%). However, following incubation of LMCs with IL-2 (100 μ/mL) a marked increase in cytotoxic activity against autologous BEC was observed (48.3 ± 9.7%). It is well known that innate immune effector cells can be activated *in vitro*

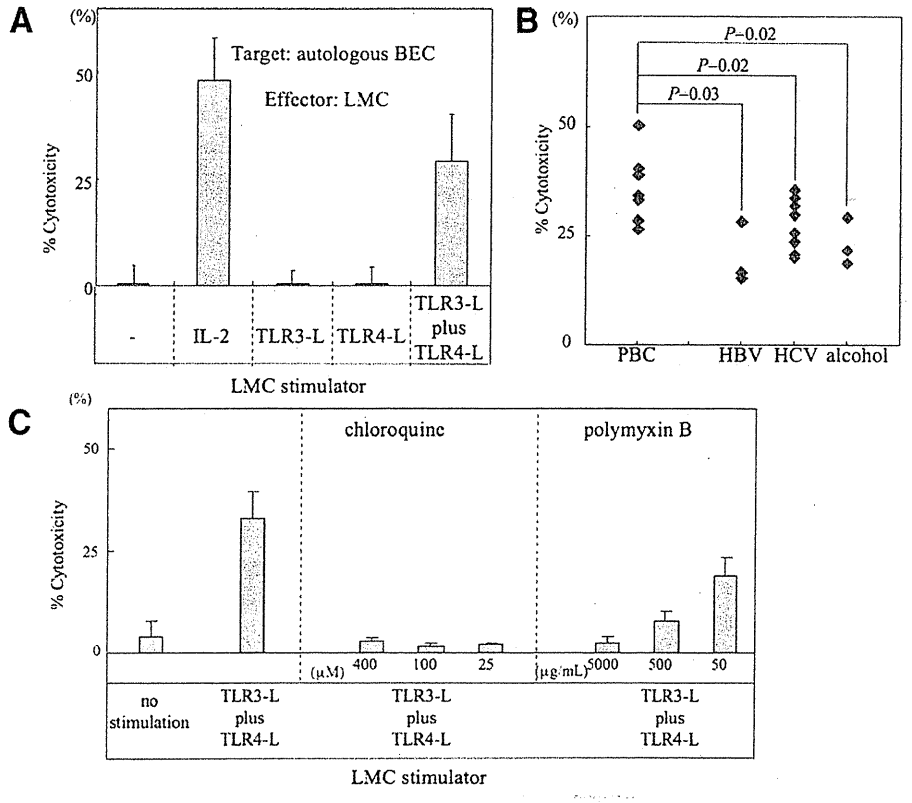

Fig. 1. (A) *In vitro* activation requirements of LMC for cytotoxicity against BEC. LMC isolated from eight patients with PBC and 14 control patients were cultured *in vitro* with either IL-2, TLR3-L alone, TLR4-L alone, or a mixture of TLR3-L+TLR4-L for 3 days and then washed and assayed for cytotoxicity against autologous BEC using the standard ^{51}Cr release assay. LMC cultured in media alone served as a negative control. The assay was performed in triplicate for each activation agent and expressed as mean ± SD. Representative data from one PBC patient are shown. (B) The net cytotoxicity for LMC against BEC was performed. There were statistical differences in the degree of net cytotoxicity induced by TLR3-L and TLR4-L activation of LMC in cells from PBC when compared to other control liver diseases. (C) The use of inhibitors of the TLR3 and TLR4 signaling pathways on the cytotoxicity of activated LMC against autologous BEC. LMC from eight PBC patients and 14 control patients were activated *in vitro* with TLR3-L+TLR4-L in the presence of various concentrations of either chloroquine (TLR3 pathway inhibitor) or polymyxin B (TLR4 pathway inhibitor) and tested for cytotoxicity against autologous BEC. The left panel shows the control cytotoxicity data of LMC cultured in media alone or following activation with TLR3-L and TLR4-L. The middle and right panels reflect data obtained on aliquots of the same LMC activated using TLR3-L and TLR4-L but cultured in the presence of chloroquine or polymyxin B, respectively. Each culture was performed in triplicate and the data shown are mean ± SD. The data shown are from one PBC patient but are representative.

by way of a number of TLR pathways besides IL-2. Thus, we studied a variety of TLR ligands either individually or in various combinations as outlined in Materials and Methods. First, whereas LMC did not demonstrate any detectable cytotoxicity against autologous BEC following ligation of any single TLR ligand (for example, the CTL activity following TLR3-L ligation was 0.5 ± 3.1% and following TLR4 ligation was 0.6 ± 3.9%) (Fig. 1A; Supporting Fig. 1A), use of the combination of TLR3-L and TLR4-L led to significant cytotoxicity against autologous BEC (CTL activity; 29.3 ± 11.1%). Importantly, LMC did not induce significant cytotoxicity against autologous BEC using any other combination of TLR ligands (Supporting Fig. 1B). To exclude the possibility that the cytotoxicity noted using the combination of TLR3-L+TLR4-L was not due to the direct effect of the TLR ligands on BEC instead of LMC, we cocultured BEC with TLR3-L and TLR4-L in a similar cytotoxic assay described above. However, no detectable cytotoxic activity was found (data not shown).

Studies were then carried out to evaluate the differences if any in the cytotoxicity of BEC following TLR3-L and TLR4-L stimulation of LMC from PBC as compared with LMC isolated from other disease controls. The net cytotoxicity of LMCs from PBC patients (n = 8) against BEC was 36.4 ± 7.5. In the case of LMCs from HBV (n = 3), HCV (n = 8), and alcohol-related cirrhosis (n = 3) controls, the net cytotoxicity was 20.2 ± 7.1, 27.7 ± 5.9, and 23.4 ± 5.5, respectively, as shown in Fig. 1B. There were statistical differences in the degree of net cytotoxicity induced by TLR3-L+TLR4-L activation of LMC in cells from PBC when compared to similarly activated LMCs

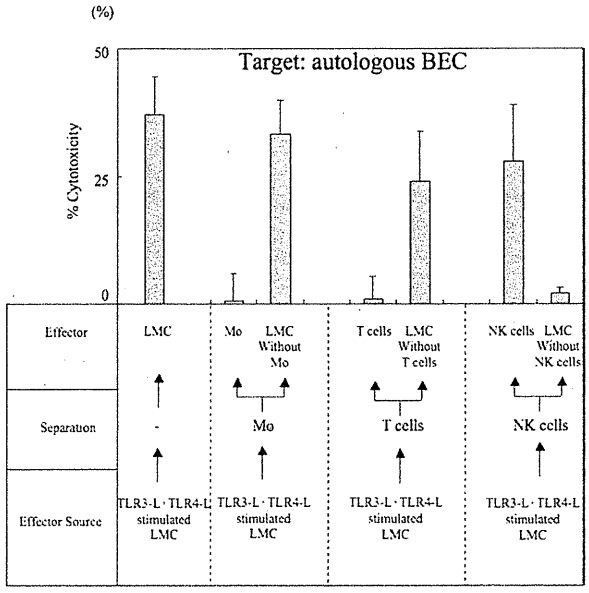

Fig. 2. Identification of the cell lineage within LMC that mediate cytotoxicity against autologous BEC. Cultures of LMC were activated *in vitro* with TLR3-L and TLR4-L and then aliquots assayed for cytotoxicity against autologous BEC (control) or used to isolate or deplete specific cell lineages. Thus, LMC were either enriched for Mo or depleted of Mo, enriched for T cells, or depleted of T cells and enriched for NK cells or depleted of NK cells and each of these tested for cytotoxicity against autologous BEC. Results of mean cytotoxicity (mean ± SD) of data obtained on one PBC patient are displayed.

from other control liver diseases (PBC versus HBV-related cirrhosis: $P = 0.03$, PBC versus HCV-related cirrhosis: $P = 0.02$, PBC versus alcohol-related cirrhosis: $P = 0.02$). Subsequently, in efforts to confirm that the activation by TLR4-L (LPS) and TLR3-L (poly I:C) was indeed induced by way of the respective TLR pathways, use was made of pretreatment of the activation agents with previously defined optimum concentrations of polymyxin B for LPS and chloroquine for poly I:C. As shown in Fig. 1C, polymyxin B inhibited CTL activity in a dose-dependent manner and chloroquine inhibited CTL activity even at the lowest concentration used.

NK Cells Are Cytotoxic for Autologous BEC in the Presence of TLR3-L+TLR4-L-Stimulated LMC. The ability of cells to induce cytotoxic activity against autologous BEC following the ligation of TLR3-L+TLR4-L was next examined. Cultures of LMC, stimulated with TLR3-L+TLR4-L, were used to either isolate enriched populations of Mo, T cells, NK cells, or isolate cultures depleted of each of these cell lineages. These enriched and depleted cell cultures were assessed for their cytotoxicity against autologous BEC. Unfractionated TLR3-L+TLR-4-activated LMC were used for purposes of a positive control. As shown in Fig. 2, whereas Mo did not demonstrate any significant cytotoxicity against autologous BEC (CTL activity; 0.6 ± 5.4%), LMC depleted of Mo demonstrated significant cytotoxicity against autologous BEC (CTL activity; 33.2 ± 6.8%). Similarly, whereas T cells did not demonstrate significant cytotoxicity against autologous BEC (CTL activity; 0.8 ± 4.5%), LMC depleted of T cells had significant cytotoxicity against autologous BEC (CTL activity; 24.0 ± 10.0%). On the other hand, whereas NK cells demonstrated significant cytotoxicity against BEC (CTL activity; 28.0 ± 11.0%), LMC depleted of NK cells did not show significant cytotoxicity against autologous BEC (CTL activity; 2.0 ± 1.1%). These data indicate that it is the NK cell lineage following TLR3-L and TLR4-L stimulation that is responsible for significant cytotoxic activity against autologous BEC. Representative data from one PBC patient is shown in Fig. 2.

TLR4-L-Stimulated NK Cells with Supernatants from TLR3-L-Stimulated LMC Are Cytotoxic for Autologous BEC. In efforts to identify the potential mechanisms by which activation of TLR3-L+TLR4-L in cultures of LMC generate cytotoxic activity of NK cells against autologous BEC, data obtained in preliminary studies showed that the activation of enriched population of NK cells with TLR3-L+TLR4-L did not lead to significant cytotoxicity against autologous BEC (Fig. 3A). These data indicate that the generation of cytotoxic activity against autologous BEC was likely due to the presence of a second population of cells. Experiments were thus carried out to clarify the relationship of NK cells, LMC, TLR3-L, and TLR4-L. We prepared supernatant fluids from LMC cultured in the presence of the appropriate ligands for either TLR3, TLR4, or TLR3+TLR4. As shown in Fig. 3A, NK cells only demonstrated cytotoxicity against autologous BEC when cultured in the presence of TLR4-L and supernatant fluids prepared from TLR3-L-activated LMC (CTL activity; 26.3 ± 11.0%), but not when cultured in the presence of TLR3-L and supernatant fluids prepared from LMC with TLR4-L (CTL activity; 0.2 ± 2.1%). The NK cells, in addition, did not kill autologous BEC in the presence of supernatant from TLR3-L and TLR4-L-stimulated LMC (CTL activity; 0.8 ± 2.8%) as shown in Fig. 3A. These data indicate that NK cells cytotoxicity against autologous BEC requires not only the activation of TLR4-L but also cytokines that are synthesized by LMC upon TLR3-L activation.

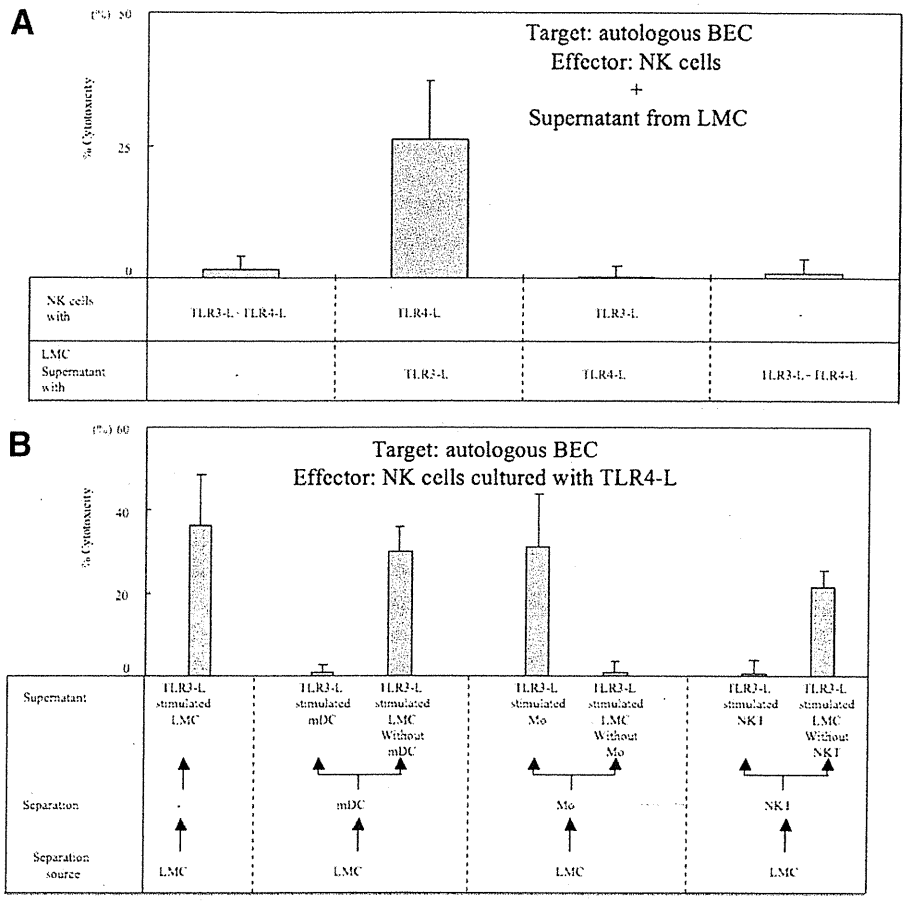

Fig. 3. (A) The activation requirements of NK cells in mediating cytotoxicity against autologous BEC. Highly enriched population of NK cells were cultured *in vitro* in the presence of (1) TLR3-L+TLR4-L; (2) TLR4-L and supernatant fluid from LMC cultured in the presence of TLR3-L; (3) TLR3-L and supernatant fluid from LMC cultured in the presence of TLR4-L; and (4) supernatant fluids from LMC cultured in the presence of TLR3-L+TLR4-L. Cultures were performed in triplicate and the mean ± SD of the net percent cytotoxicity calculated. The data shown are from one PBC patient and are representative. (B) Identification of the cell lineage that is the source of the factor required to mediate cytotoxicity of autologous BEC by TLR4-L-activated NK cells. A pool of a highly enriched population of NK cells was cultured with TLR4-L in the presence of supernatant fluids from (1) unfractionated LMC cultured with TLR3-L (control); (2) highly enriched populations of mDC or LMC depleted of mDC-stimulated with TLR3-L; (3) highly enriched population of Mo or LMC depleted of Mo-stimulated with TLR3-L; and (4) highly enriched population of NKT cells to LMC depleted of NKT cells stimulated with TLR3-L. These cultures were tested for cytotoxicity against autologous BEC. Each culture was performed in triplicate and the data shown reflect mean ± SD of net percent cytotoxicity of the triplicate cultures. The data shown are from one PBC patient and are representative.

Supernatant from TLR3-L-Stimulated Mo Induces NK Cell Cytotoxicity. We next carried out studies in efforts to identify the cell lineage that was the source of the cytokine(s) in the supernatant fluids from TLR3-L-activated unfractionated LMC that induced TLR4-L-stimulated NK cell cytotoxicity against autologous BEC. Highly enriched populations of mDC, Mo, NKT cells, and the corresponding population of LMCs depleted of mDC, Mo, and NKT cells were stimulated with TLR3-L and the supernatant harvested; insufficient quantities were available to study the pDC fraction. NK cells were cultured with TLR4-L in the presence or absence of each of these supernatant fluids and analyzed for cytotoxicity against autologous BEC as described in Materials and Methods. As noted in Fig. 3B, whereas TLR4-L-stimulated NK cells cultured in the presence of supernatant fluids from TLR3-L unfractionated LMC demonstrated significant cytotoxicity; similarly TLR4-L-stimulated NK cells, when cultured with supernatant fluids of TLR3-L, stimulated mDC, and NKT cells did not demonstrate detectable cytotoxicity against autologous BEC. However, the TLR4-L-activated NK cells, cultured in the presence of TLR3-L-activated Mo, readily demonstrated cytotoxicity. The identification of Mo as the source of the cytokine required for TLR4-L-activated NK cells to induce cytotoxicity against autologous BEC was confirmed by results obtained with

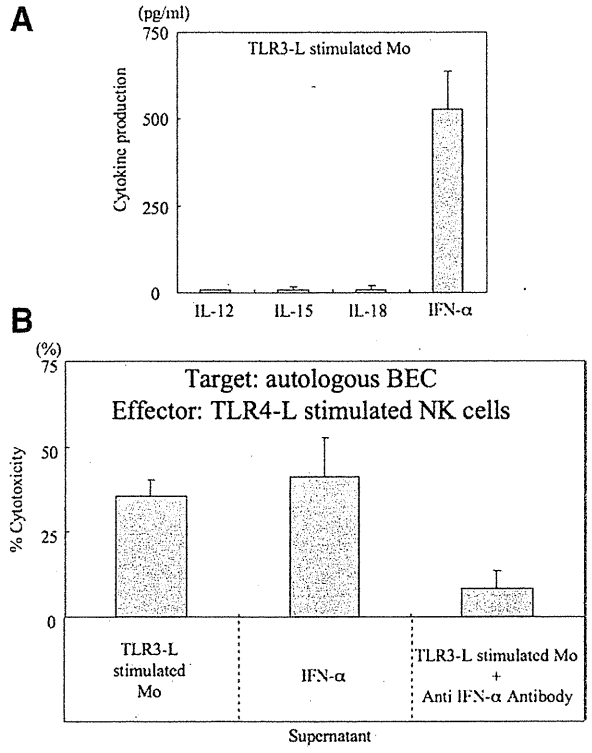

Fig. 4. (A) Analysis of cytokines synthesized by *in vitro* TLR3-L-activated hepatic Mo. Mo from the liver of the 22 patients included in the present study were isolated and cultured *in vitro* and the supernatant fluids analyzed for levels of IL-12, IL-15, IL-18, and IFN-α. Cultures were performed in triplicate and the data displayed represents mean ± SD of values obtained from cultures from one representative patient. Statistical differences between PBC patients and disease controls are described in the text. (B) IFN-α is required by TLR4-L-stimulated NK cells to mediate cytotoxicity against autologous BEC. Aliquots of TLR4-L-stimulated NK cells were cultured in the presence of either supernatant fluids from TLR3-L-stimulated hepatic Mo (control), IFN-α, or supernatant fluids from TLR3-L-stimulated Mo incubated with previously determined optimum concentration of anti-IFN-α monoclonal antibody. Cultures were performed in triplicate and assayed for cytotoxicity against autologous BEC. Data displayed are net percent cytotoxicity and the data shown are from one representative PBC patient.

supernatant fluids from TLR3-L-stimulated LMC depleted of mDC, and NKT cells, respectively.

TLR3-L-Stimulated Mo Produce IFN-α. The nature of the cytokine synthesized by TLR3-L-activated Mo that promoted cytotoxicity in TLR4-L-activated NK cells was studied next. We reasoned that the cytokine responsible for this activity was most likely IL-12, IL-15, IL-18, or IFN-α, which have previously been shown to generally activate NK cells. As seen in Fig. 4A, whereas TLR3-L-stimulated Mo produced low but detectable levels of IL-12 (7.9 ± 3.4 pg/mL), IL-15 (9.8 ± 8.0 pg/mL), and IL-18 (10.0 ± 9.6 pg/mL), the major cytokine synthesized was shown to be IFN-α (530.1 ± 106.2 pg/mL). In efforts to confirm that it was indeed IFN-α that was responsible for inducing TLR4-L-activated NK cell cytotoxicity, aliquots of TLR4-L-activated NK cells were cultured in the presence or absence of various concentrations of either IL-12, IL-18, IL-15, or IFN-α (Fig. 4A). Data derived from such studies demonstrated that whereas TLR4-L-activated NK cells cultured in the presence of IL-12, IL-18, or IL-15 (10-20 pg/mL) had no detectable cytotoxicity (data not shown), TLR4-L-activated NK cells cultured in the presence of recombinant IFN-α (500 pg/mL) readily induced cytotoxicity against autologous BEC (cytotoxicity; 41.2 ± 11.4%) (Fig. 4B). The identity of IFN-α as the cytokine responsible for inducing cytotoxicity in cultures of TLR4-L-activated NK cells was confirmed with the use of anti-IFN-α antibody. Thus, pretreatment of supernatant fluids from TLR3-L-activated Mo with anti-IFN-α reduced the cytotoxicity of TLR-4-stimulated NK cells against autologous BEC (cytotoxicity; 8.5 ± 5.2%). We also examined the relative levels of IFN-α synthesized by TLR3-L-activated Mo from patients with other diseases as compared with Mo from PBC patients in efforts to determine whether there was a qualitative and/or quantitative difference in the synthesis of this cytokine. IFN-α production from TLR3-L-activated Mo from PBC patients (n = 8; 355 ± 132 pg/mL) was significantly higher than similarly activated Mo from HBV-related cirrhosis (n = 3; 175 ± 74 pg/mL: $P < 0.03$), HCV related cirrhosis (n = 8; 175 ± 57 pg/mL: $P < 0.01$), or those from alcohol-related cirrhosis (n = 3; 180 ± 54 pg/mL: $P < 0.03$).

Contribution of Other Molecules to Liver NK Cell Cytotoxicity Against Autologous BEC. Although the above studies identified IFN-α as the cytokine synthesized by TLR3-L-activated Mo, we next attempted to identify the nature of the molecules synthesized by NK cells that were potentially involved in mediating cytotoxicity against autologous BEC. First, we evaluated the expression of activating receptors, inhibitory receptors, and effectors using reverse transcriptase (RT)-PCR methods on mRNA isolated from unstimulated NK cells, TLR4-L-stimulated NK cells, IFN-α-stimulated NK cells, and the combination of TLR4-L and IFN-α-stimulated NK cells. As shown in Fig. 5A, based on the activation signals the cultured cells expressed effector molecules such as FasL, TRAIL, and/or Granzyme B. Among these effector molecules, TRAIL appeared to be the molecule involved in promoting the cytotoxicity of TLR4-L-activated NK cells. Thus, as shown in Fig. 5B, the addition of monoclonal anti-TRAIL antibody but not anti-FasL antibody or anti-Granzyme B significantly reduced the cytotoxicity