Increased activated natural killer T cells in the liver of patients with advanced stage primary biliary cirrhosis

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ABSTRACT

Although growing evidence suggests a major role for T cells in the pathogenesis of primary biliary cirrhosis (PBC), the roles of natural killer (NK) and natural killer T (NKT) cells, which predominate in the liver, in the pathogenesis of PBC remain unclear. We investigated the status of NK and NKT cells in the liver and peripheral blood samples obtained from 11 patients with asymptomatic PBC diagnosed as stage I or II (early PBC) and 7 patients with symptomatic PBC who underwent liver transplantation (advanced PBC) using flow cytometry and immunohistochemical staining. The proportions of NK and NKT cells were significantly decreased in the liver of patients with early PBC compared with normal donors. However, the proportion of CD56⁺ NKT cells was increased in the liver of patients with advanced PBC. Moreover, the proportion of activated Fas ligand (FasL)-positive NKT cells was significantly increased in the liver of patients with advanced PBC compared with early PBC (P = 0.013). We also found increased expression of FasL on lymphocytes infiltrating around the injured bile duct in advanced PBC using immunohistochemical staining. Our results suggest that activated NKT cells may contribute to the biliary epithelial cell death resulting in the progression of PBC.

Primary biliary cirrhosis (PBC) is a chronic progressive cholestatic liver disease characterized by immunemediated destruction of small- and medium-sized intrahepatic bile ducts and the presence of anti-mitochondrial antibodies (AMA) in the sera of affected patients (12, 19). PBC is considered a model of autoimmune diseases based on several features, including the presence of a highly direct and very specific immune response to mitochondrial autoantigens, female predominance, and homogeneity among patients (24). Because the mitochondria exists in virtually all nucleated cells, the mitochondrial autoantigens are ubiquitous proteins expressed in all nucleated cells. However, the immunopathology of PBC is charac-
characterized by the presence of CD4+ and CD8+ T-cell infiltrates in the liver and targeted destruction of biliary epithelial cells (10, 13). Although the role of adaptive immune responses has been extensively studied, the pathogenesis of PBC remains obscure. As it is becoming increasingly clear that the innate immune response plays a prominent role in regulating the quality of the adaptive immune response that ensues (14, 22), the importance of innate immunity in the pathogenesis of PBC is now apparent (2).

Among various innate immune cells, natural killer T (NKT) cells, which can be distinguished by their co-expression of T cell receptors (TCR) and various natural killer (NK) cell markers, represent a heterogeneous population of immunoregulatory and effector cells (18). Functionally, a major subset of these cells is defined by reactivity against the non-polymorphic major histocompatibility complex (MHC) class I-like molecule, CD1d, and NKT cells are classified based on CD1d restriction as invariant NKT (iNKT) or non-iNKT cells (14, 18, 22). The classical iNKT cell population, which is also referred to as type I NKT cells, expresses a highly restricted TCR repertoire (Vα14-Jα18 paired with Vβ8.2, 7, or 2 in mice or the homologous Vα24-Jα18 paired with Vβ11 in humans) (25). In contrast, non-iNKT cells, also referred to as type II NKT cells, express more diverse TCR than iNKT cells. These cells possess important effector functions in immunity against cancer and microbial pathogens, but their immunoregulatory function has recently received much attention (18). In particular, NKT cells are enriched in the liver and have been reported to play a diverse role in acute liver injury, liver fibrosis, and tolerance (8, 14). Although iNKT cells represent up to 50% of rodent intrahepatic lymphocytes, human liver contains few classical iNKT cells (<1%). Instead, NKT cells in the human liver are rich in CD3+CD56+ non-iNKT cells (6). NKT cells have been reported to play an important role in the exacerbation of disease in murine PBC models (4, 20, 26), but the precise role of NKT cells in the pathogenesis of PBC remains unclear. To address this issue, we focused on NKT cells, particularly CD56+ NKT cells, and investigated the changes of these cells in the liver of patients with PBC during disease progression using biopsied or resected liver samples. Because of the limited numbers of cells obtained from liver biopsy samples, it was difficult to sufficiently analyze events to acquire reliable data on the small population of iNKT cells. Therefore, we used CD16, CD56, and CD161, which have been employed in many previous studies, to define non-iNKT cells rather than iNKT cells.

MATERIALS AND METHODS

Study cohort. This work was conducted in accordance with the Declaration of Helsinki. Written informed consent under institutional review board-approved protocols at Niigata University Hospital between 2000 and 2012. The diagnosis of PBC was based on clinical and laboratory data and confirmed by histopathological examination of the liver. Eleven patients (9 females and 2 males) who underwent needle liver biopsies were diagnosed as stage I or II (early PBC). Their ages ranged from 29 to 75 years (mean 56.9 years). Seven patients (5 females and 2 males) who underwent LDLT were diagnosed as stage IV (advanced PBC), and their ages ranged from 46 to 60 years (mean 51.4 years). Seven healthy donors (5 females and 2 males) for LDLT served as controls. Their ages ranged from 19 to 27 years (mean 22.3 years). Additional clinical data are shown in Table 1.

Reagents. Monoclonal antibodies (mAb) used were anti-CD3 (UCHT1), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-CD16 (3G8), anti-CD28 (CD28.2), anti-CD45 (2D1), anti-CD56 (B159), anti-CD57 (HNK-1), anti-CD69 (FN50), anti-CD152 (BN13), anti-CD161 (DX12), and anti-CD95 ligand (CD95L) (NOK-1) (BD Biosciences, San Diego, CA). Anti-CD56 (MOC-1) and anti-CD95L (G247-4) antibodies for immunohistochemical staining were purchased from DAKO (Glostrup, Denmark) and BD Biosciences, respectively. Control murine IgG2a and IgG1 were purchased from BD Biosciences.

Cell isolation. Liver tissues were minced into pieces in Eagle’s minimum essential medium (MEM) (GIBCO/Life Technologies, Grand Island, NY) supplemented with 5 mM HEPES (Sigma Chemical Co., St. Louis, MO), 0.1% collagenase and 0.01% Trypsin inhibitor (Sigma). The resultant finely minced liver tissue was stirred gently in presence of enzymes in a 37°C water bath for 15 min. The enzymatically digested liver tissue was pressed through a 200-gauge stainless steel mesh, washed twice, and suspended in MEM containing of 5 mM HEPES and 5% heat-inactivated fetal calf serum. Liver cell suspensions were overlaid on Ficoll-Paque (GE Healthcare Bio-Sciences, Uppsala, Sweden) and centrifuged.
NKT cells in the liver of PBC

Table 1  Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Normal donors</th>
<th>Early PBC</th>
<th>Advanced PBC</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>22.3 ± 3.0</td>
<td>56.9 ± 12.7</td>
<td>51.4 ± 5.8</td>
</tr>
<tr>
<td>ALP (IU/mL)</td>
<td>WNR</td>
<td>386.7 ± 112.4</td>
<td>641.1 ± 248.2***</td>
</tr>
<tr>
<td>T.Bil (mg/dL)</td>
<td>WNR</td>
<td>0.7 ± 0.3</td>
<td>12.1 ± 6.4*</td>
</tr>
<tr>
<td>IgM (mg/dL)</td>
<td>WNR</td>
<td>340.0 ± 168.1</td>
<td>659.0 ± 318.4***</td>
</tr>
<tr>
<td>AMA (+/−)</td>
<td>0 / 7</td>
<td>9 / 2</td>
<td>6 / 1</td>
</tr>
<tr>
<td>Scheuer stage</td>
<td>1 / 11 = 8 / 3</td>
<td>7 / 1</td>
<td>7</td>
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Abbreviations: PBC, primary biliary cirrhosis; ALP, alkaline phosphatase; T.Bil, total bilirubin; AMA, anti-mitochondrial antibodies; WNR, within normal range. Mean ± SD. *P < 0.01, **P < 0.05 (compared with early PBC).

RESULTS

NK and NKT cells in the liver and peripheral blood of patients with PBC

The total cell numbers of intrahepatic MNC were significantly higher in the patients with PBC (1.4 ±

**Immunofluorescence analysis.** Cell surface Ag expression was determined by a three-color immunofluorescence assay. Cells (10^5) were labeled with fluorescein isothiocyanate (FITC)-, phycoerythrin- or CyChrome-conjugated mAbs. Cells were incubated with the appropriate mAbs for 30 min at 4°C in PBS with 0.1% FCS and 0.02 mM NaN3. Cytoplasmic cytotoxic T-lymphocyte antigen 4 (CTLA-4) was determined by intracellular flow cytometry as follows. After surface staining, cells were permeabilized using Cytofix/Cytoperm solution (BD Biosciences), stained with PE-anti-CD152 (CTLA-4) mAb (BD Biosciences) for 30 min at 4°C and washed twice. After wash, the labeled cell samples were analyzed on a FACScan or FACS Calibur (Becton Dickinson, San Jose, CA). PBMC and hepatic MNC were analyzed with CD45^+ cell gating to exclude non-MNC.

**Immunohistochemistry.** The liver specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections 5 μm thick were cut and mounted. The slides were deparaffinized with three changes of xylene each for 10 min, followed by successive changes of 100%, 80% and 70% ethanol each for 5 min. For antigen retrieval the slides were pretreated by immersion in 10 mM sodium citrate buffer, pH 3.5 and 0.01 (w/v) EDTA (BioGenex, San Ramon, CA) at 95°C for 10 min and allowed to cool in the buffer for 20 min. Following 3 washes in PBS for 5 min each, endogenous peroxidase was blocked with 0.3% H2O2 in methanol for 30 min at room temperature. After immersion in blocking serum for 30 min, sections were incubated at 4°C overnight with a mouse monoclonal anti-human FasL (BD Biosciences) or anti-human CD56 antibody (DAKO) at a 1 : 400 or 1 : 100 dilution, respectively, in PBS supplemented with 3% bovine serum albumin. After successive washing in PBS, sections were incubated with biotinylated anti-mouse immunoglobulin at a 1 : 100 dilution in PBS supplemented 5% bovine serum albumin. Statistical analysis. The significance of differences was analyzed statistically by Fisher’s exact test, the compared t test with Welch’s correction or Mann-Whitney U test using SPSS software (Ver.18, SPSS Inc., Chicago, IL). The level of significance was set at P < 0.05.
Expression of CD28 and CD152 on CD56+ NKT cells

CD28 is one of the co-stimulatory molecules expressed on T and NKT cells, and CD28/B7 co-stimulatory pathways have been shown to be important for regulation of NKT cells as well as T cells (1, 3). CD152 (CTLA4) is structurally as well as functionally related to CD28, and binds to the B7 family molecules CD80 and CD86 with higher affinity (17). After activation of NKT cells, CD152 (CTLA4) is up-regulated, and provides negative co-stimulatory signals (1, 3, 27). We observed that most CD56+ NKT cells in the liver of normal donors expressed CD28 (79.7 ± 15.2%), but in the patients with both early and advanced PBC intrahepatic CD56+ NKT cells significantly decreased the expression of CD28 (P < 0.01) (Fig. 3A). Most CD3+CD56− T cells in the liver also expressed CD28 (78.6 ± 10.8%), and no significant differences were observed between the normal donors and patients with PBC.

In PBL, approximately half of CD56+ NKT cells expressed CD28 (41.6 ± 24.9%) in the normal donors, but in the patients with both early and advanced PBC, CD56+ NKT cells significantly decreased the expression of CD28 (both P < 0.01) (Fig. 3B). Most CD3+CD56− T cells in PBL expressed CD28 (90.3 ± 10.3%), and no significant differences were observed between the normal donors and patients with PBC (Fig. 3B).

CD152 expression on CD56+ NKT cells in the liver was also significantly lower in the patients with both early and advanced PBC (2.5 ± 0.9 × 10^6/g liver) than the normal donors (1.1 × 10^7/g liver) (P < 0.01). However, no significant differences were observed in the total cell numbers of PBL between PBC patients and normal donors (data not shown). To identify the phenotype of lymphocytes, three-color staining for CD3, NK cell markers (CD16, CD56, CD57, CD161), and various markers were conducted (Figs. 1 & 2). The proportion of CD56+ NKT cells in the liver of patients with early PBC was significantly lower than in the normal donors (P = 0.034), but the proportion of CD56+ NKT cells in the liver of patients with advanced PBC was increased and significantly higher than in the patients with early PBC (P = 0.035) (Fig. 2A). CD57+ NKT cells in the liver also constituted a significantly higher proportion in advanced PBC compared with normal donors (P = 0.021). Significantly higher proportions of conventional CD3+CD56− T cells were observed in the liver of patients with early PBC (58.5 ± 10.2%) compared with normal donors (35.7 ± 8.3%) (P < 0.01); however, decreased proportions were observed in advanced PBC (46.5 ± 16.0%) compared with early PBC patients. The proportion of NK cells tended to decrease in the liver of patients with PBC (Fig. 2A), while the proportion of NK cells in the PBL was not significantly different between the normal donors and patients with PBC (Fig. 2B).

Fig. 1 Phenotypic characterization of hepatic lymphocytes using flow cytometry. Three-color staining for CD3, NK cell markers (CD16, CD56, CD57, CD161), and various markers were conducted in the normal donors and patients with early and advanced PBC. Numbers in the figure represent the percentages of fluorescence-positive cells in corresponding areas. Representation data are shown.
NKT cells in the liver of PBC

er of patients with PBC increased with the progression of disease and was significantly higher in both early and advanced PBC than in the normal donors (both $P < 0.01$, respectively) (Fig. 3A). A significant difference was also observed in the expression of CD152 on CD56$^+$ NKT cells in the liver of patients with early and advanced PBC ($P < 0.01$). These results suggest that CD152$^+$ activated CD56$^+$ NKT cells increased with the progression of PBC. No significant differences were observed in CD152 expression on CD3$^+$CD56$^-$ cells between the normal donors and patients with PBC (Fig. 3A). In PBL, no significant differences were observed in CD152 expression between CD56$^+$ NKT and CD3$^+$CD56$^-$ T cells (Fig. 3B).

Expression of Fas ligand on CD56$^+$ NKT cells

Little expression of FasL on CD56$^+$ NKT cells in the liver was observed in the normal donors (1.4 ± 0.4%); however, in the patients with both early and advanced PBC, intrahepatic CD56$^+$ NKT cells exhibited significantly increased expression of FasL (7.4 ± 4.5% and 31.1 ± 19.2%, respectively) ($P < 0.01$ and $P = 0.013$, respectively) (Fig. 4A). We also studied the expression of FasL on CD3$^+$CD56$^-$ T cells and CD3$^+$CD56$^-$ NK cells in the liver, but no significant differences were observed in the expression of FasL on the CD3$^+$CD56$^-$ T cells and CD3$^+$CD56$^-$ NK cells with the progression of PBC (Fig. 4A). In PBL, no significant changes in FasL expression on CD56$^+$ NKT cells, CD3$^+$CD56$^-$ T cells, and CD3$^+$CD56$^-$ NK cells were observed (Fig. 4B).

Immunohistochemical detection of FasL

We then conducted immunohistochemical staining in the liver of patients with PBC. In the liver of patients with early PBC, FasL expression was occasionally found on the infiltrating MNC within the portal tracts (Fig. 5A). However, in the liver of patients with advanced PBC, distinct expression was observed on MNC infiltrating around the injured bile duct (Fig. 5B). Close contact of FasL-positive MNC with the biliary epithelial layers was also observed (Fig. 5B).
We conducted additional immunohistochemical staining at both early and advanced stages in the liver of a single patient with PBC who underwent liver transplantation, confirming that the expression of FasL on MNC was more abundant in the liver at the advanced stage (Scheuer stage IV) than the early stage (Scheuer stage II) (Fig. 5C, D).

DISCUSSION

In the present study, we found that the proportions of NK cells and CD56+ NKT cells in the liver of patients with early PBC were significantly decreased compared with normal donors, but the proportion of CD56+ NKT cells in the liver of patients with advanced PBC was significantly higher than in patients with early PBC. Among the increased proportion of CD56+ NKT cells in the liver of patients with advanced PBC, activated FasL+ NKT cells were observed at a significantly high proportion. Furthermore, we confirmed that the expression of FasL on MNC was more abundant in the liver at advanced stages than early stages using immunohistochemical staining in a single patient with PBC who underwent liver biopsy at the early stage and liver transplantation at the advanced stage. Our results suggest that NKT cells may play an important role in the progression of PBC. Moreover, the decrease of NK and NKT cells in the liver of patient with early PBC may be associated with the breakdown of self-tolerance against target cells in PBC because increasing evidence suggests that recognition of self-antigens by NKT cells plays an important role not only in their development but also in their regulation of a broad range of immune responses (9).
NKT cells in the liver of PBC

The lymphocyte subsets expressing FasL in the liver of patients with PBC have not been elucidated. Because it has been reported that FasL is upregulated in cytotoxic T cells, Th1 subset of helper T cells, and NK cells, as well as NKT cells (23), we analyzed the expressions of FasL on CD3⁺CD56⁻ T cells, CD3⁺CD56⁺ NK cells, and CD3⁺CD56⁺ NKT cells. In the present study, we observed no significant change in the FasL expression on CD3⁺CD56⁻ T cells or CD3⁺CD56⁺ NK cells with the progression of disease. However, the expression of FasL on CD56⁺ NKT cells in the liver increased with the progression of disease and was significantly higher in both early- and advanced-stage PBC than in the normal donors. Our results suggest that NKT cells may play an important role in the progression of bile duct and hepatocyte injury in PBC through Fas/FasL interaction.

NKT cells have been reported to play an important role in the exacerbation of disease in murine PBC models. Mattner et al. reported that iNKT cell activation upon infection by Novosphingobium aromaticivorans initiates liver injury, although it will be difficult to interfere in established PBC by modulating iNKT cell function (15). In the transforming growth factor beta (TGF-β) receptor II dominant-negative murine model of PBC, the activation of iNKT cells has been shown to be a critical factor in acceleration of the disease (4). In a xenobiotic induced model of PBC, autoimmune cholangitis is induced by immunization of mice with 2-octynoic acid coupled to bovine serum albumin, an antigen selected following quantitative structure-activity relationship analysis of the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), which is the immune-dominant autoantigen of PBC (26). Wu et al. reported that activation of iNKT cells stimulated with alpha-galactosylceramide (α-GalCer) resulted in a profound exacerbation of autoimmune cholangitis in the xenobiotic murine PBC model (26). Shimoda et al. also reported that in vivo depletion of NK and NKT cells markedly suppressed the production of AMA and cytokines from autoreactive T cells in a xenobiotic murine model of PBC (20). Although such important roles of NKT cells in the exacerbation of disease in murine PBC models have been clearly indicated, there have been only a limited number of reports analyzing NKT cells in the liver of patients with PBC. Because we could obtain only a limited number of liver samples and a very small number of cells from the liver biopsy samples, we included some data from previously unpublished results of several patients who underwent liver biopsy.

Fig. 4  Expression of Fas ligand on T, NK, and NKT cells in the liver and peripheral blood of normal donors and patients with PBC. Expression of Fas ligand (FasL) on CD3⁺CD56⁻ T cells, CD3⁺CD56⁺ NK cells, and CD3⁺CD56⁺ NKT cells in the liver (A) and peripheral blood (PB) (B) of normal donors and patients with PBC were analyzed using flow cytometry. The percentages of FasL⁺ cells among the indicated subsets are shown. The mean ± SD is presented. *P < 0.01 (compared with normal donors), **P < 0.01 (compared with early PBC).

Previously reported changes of activated NKT cells in the liver of patients with chronic viral hepatitis (27, 28). Activated NKT cells can induce hepatocyte and biliary epithelial cell death directly through up-regulation of cell surface FasL expression and/or the release of tumor necrosis factor alpha (TNF-α) and perforins/granzyme B, or indirectly through the release of interferon-gamma (IFN-γ) (8, 18, 21). Consistent with our findings that FasL expression on MNC was abundant in the liver of patients with PBC, Harada et al. reported that MNC infiltrating around the injured bile ducts of PBC frequently expressed FasL (11). Moreover, they also reported that apoptotic cells were frequently found on biliary epithelial cells in PBC, and the interlobular bile ducts in patients with PBC frequently expressed CD95 (Fas) (11). Those findings suggest that enhanced apoptosis mediated by Fas/FasL interaction may contribute to the injury and loss of bile ducts observed in the liver of patients with PBC. However,
Therefore, we recognize that the present study appears to be somewhat outmoded and has several limitations, including a small number of enrolled patients, a limited number of investigated molecules associated with the activation and killing activity of NKT cells, and no assays of function. However, we believe that the present study may provide important information on the role of NKT cells in the pathogenesis of PBC.

In conclusion, to the best of our knowledge, we have demonstrated for the first time that the proportions of NK and NKT cells decreased significantly in the liver of patients with early PBC compared with normal donors and that activated FasL-positive NKT cells were significantly increased in the liver of patients with advanced PBC compared with early PBC. Further precise investigations to reveal the characteristics of NKT cell increases in the liver of patients with advanced PBC should be conducted, but our results may provide new insights into the role of innate lymphoid cells, particularly NKT cells, in the mechanism of disease progression of PBC.

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