Progressive familial intrahepatic cholestasis (PFIC), an inherited autosomal recessive liver disease, is characterized primarily by persistent intrahepatic cholestasis and jaundice in the first year of life. Its actual prevalence remains unknown, but its estimated incidence varies between 1/50,000 and 1/100,000 births. This disease is classified as a rare hepatic disorder, with an estimated incidence of 1/250,000 births in the general population. PFIC is a genetic disorder, and its diagnosis is often made after birth, when symptoms first appear. It is due to mutations in the ATP8B1 gene, which leads to the dysfunction of the canalicular ATPase, a protein responsible for the secretion of bile into the bile ducts. The deficiency of this enzyme results in the accumulation of bile acids in the liver, leading to the development of cholestasis and jaundice. The diagnosis of PFIC is usually made using a combination of clinical, biochemical, and genetic tests. The treatment of PFIC is supportive and aimed at managing the symptoms and complications of the disease. In some cases, liver transplantation may be necessary. The prognosis of PFIC is variable, and patients may experience a range of outcomes, from a mild disease to a severe and progressive liver failure. The identification of patients with PFIC is essential for early intervention and management, which can improve the quality of life and survival of affected individuals. The research aim is to develop a novel diagnostic method for PFIC, which could be used to identify patients with the disease and their subtypes, even before the onset of symptoms. The research hypothesis is that the expression of ATP8B1 is altered in patients with PFIC, and that this alteration can be detected using peripheral blood monocyte-derived macrophages (PBMDCs). The research objective is to develop a diagnostic method for PFIC based on the analysis of ATP8B1 expression in PBMDCs. The study is designed to test the hypothesis that the expression of ATP8B1 is altered in patients with PFIC, and that this alteration can be detected using PBMDCs. The study is expected to be conducted in a large cohort of patients with PFIC, including patients with different subtypes of the disease. The study will be conducted in collaboration with experts in the field, and the results will be published in a peer-reviewed journal.
ATP8B1, but not on its transport activity, even though the existence of specimens can evaluate the impact of the mutation on the expression of nonsense, frameshift, and large deletion mutations, which occur in ATP8B1 expression in liver biopsy specimens. However, genomic analysis of the sequencing plan, it is of the highest priority to develop a methodology to identify as disease-causing mutations or rare normal variants (Davit-Spraul et al., 2010; Klomp et al., 2004; Liu et al., 2007; Matte et al., 2010). This, together with the fact that individuals with normal-GGT PFIC share many clinical features (Davit-Spraul et al., 2009), makes the determination of the subtypes of PFIC more complex.

Normal-GGT PFIC progresses to severe cholestasis with sustained intractable itching, jaundice, and failure to thrive, resulting in liver failure and death before adulthood (Morotti et al., 2011; Suchy et al., 2014). Although we and other groups have reported that 4-phenylbutyrate improves the intractable itching in patients with PFIC1 (Hasegawa et al., 2014) and the biochemical parameters and liver histology in patients with PFIC2 (Gonzales et al., 2012; Hayashi and Sugiyama, 2007; Hayashi et al., 2005; Naoi et al., 2014), no effective medical therapy for this disease has been established (Morotti et al., 2011, Suchy et al., 2014). Currently, the only curative options for normal-GGT PFIC are surgical procedures including liver transplantation (LTx) (Suchy et al., 2014). However, while LTx solves the immediate problem of liver failure in PFIC1, it is insufficient to overcome an ATP8B1 deficiency because of ongoing steatosis and fibrosis (Hori et al., 2011; Miyagawa-Hayashino et al., 2009). Therefore, to understand the potential of a possible treatment plan, it is of the highest priority to develop a methodology to identify PFIC1 patients correctly at an early age of their disease course.

The current procedure for diagnosing PFIC1 is to analyze the sequence of ATP8B1 and, if possible, to confirm the hepatic level of ATP8B1 expression in liver biopsy specimens. However, genomic analysis is insufficient to make a definitive diagnosis of PFIC1 except in cases of nonsense, frameshift, and large deletion mutations, which occur in 40% of patients who carry ATP8B1 mutations in both alleles (Davit-Spraul et al., 2010; Klomp et al., 2004). Studies with liver biopsy specimens can evaluate the impact of the mutation on the expression of ATP8B1, but not on its transport activity, even though the existence of mutations that affect the transport activity of ATP8B1 has been suggested (Folmer et al., 2009; van der Velden et al., 2010). In addition, liver biopsy is invasive and involves a high risk of complications such as bleeding, pneumothorax, and pain. Therefore, it is desirable to develop a diagnostic method to evaluate the function of ATP8B1 using specimens that can be collected less invasively.

In the current study, we explored blood cell populations that endogenously express ATP8B1 and detected its expression in human peripheral blood monocyte-derived macrophages (HMDM). Of the various subpopulations of HMDM, ATP8B1 was predominantly expressed in M2c, a subset of alternatively activated macrophages that is induced by exposure to interleukin-10 (IL-10) and whose function is related to suppression of immune responses and tissue remodeling (Mantovani et al., 2004). The impact of impaired function of ATP8B1 in M2c was examined by flow cytometric and microscopic analysis using HMDM transfected with siRNA against ATP8B1, and then confirmed using HMDM from PFIC1 patients. Based on the phenotypes identified in this analysis, we tested whether ATP8B1 function was impaired or maintained in patients with a clinical diagnosis of normal-GGT PFIC in whom only one mutant allele of ATP8B1 was detected by genome sequencing (patients with PFIC1-like disease). The results were verified by evaluating expression of ATP8B1 in liver specimens from the patients and by an in vitro mutagenesis study.

2. Materials and Methods

A detailed description of the materials and methods is presented in the Supporting information. All methods used standard techniques and commercially available reagents.

2.1. Sampling of Blood and Liver Specimens from Patients and Control Subjects

This study was approved by the institutional ethics review board at the University of Tokyo and was performed in accordance with the principles stated in the Declaration of Helsinki. Before assessment of subjects, they or their parents (when the subjects were under 18 years of age) provided signed informed consent.

The clinical diagnosis of normal-GGT PFIC was based on the presence of unremitting hepatocellular cholestasis with intractable pruritus, poor growth, jaundice with conjugated hyperbilirubinemia, elevated serum bile acid concentrations, and normal serum GGT level, in the first year of life. Serological, viral, or metabolic markers, imaging, and urine screening were performed to exclude other causes of cholestasis including hepatitis B and C virus (HBV and HCV) infections, inborn errors in bile-acid synthesis, and ductal origin. In patients with a clinical diagnosis of normal-GGT PFIC, ATP8B1 and ABCB11 were analyzed by Sanger sequencing, as described previously (Hasegawa et al., 2014; Naoi et al., 2014). The patients in whom mutations in both alleles of either gene were undetectable by Sanger sequencing were subjected to targeted next-generation sequencing that analyzed genes known to be responsible for neonatal/infantile intrahepatic cholestasis (ATP8B1, ABCB11, ABCB4, TJP2, JAG1, NOTCH2, ABC2, SLCA5A3, HSD3B7, AKR1D1, CYP7B1, VPS33B, BAAT, EPHX1, SLCO1A1) (Togawa et al., 2016).

Peripheral blood samples from patients with normal-GGT PFIC and age-matched controls including healthy individuals, obese individuals, and those with pancreatitis, HBV, HCV, Alagille syndrome, citrininemia, and biliary atresia were collected in EDTA-2 K-coated blood sampling tubes (Becton Dickinson, Tokyo, Japan) for isolation of human peripheral blood monocytes (HPBMo) and their subsequent differentiation into human monocyte-derived macrophages (HMDM). In each assay except for Fig. 1c in which interindividual variability was examined in HMDM, the monocytes pooled from more than three control subjects were differentiated into HMDM and then used to identify blood cell populations expressing ATP8B1 and to examine the impact of ATP8B1 in HMDM.
employed as control cells to evaluate HMDM from each patient with normal-GGT PFIC.

A liver sample from the patients with normal-GGT PFIC and the age-matched control subjects (those with HBV, HCV, or Alagille syndrome) was obtained by biopsy for diagnosis or at the time of LTx, snap-frozen in liquid nitrogen, and stored at −80 °C. The membrane fractions from these specimens were prepared as described previously (Hasegawa et al., 2014; Naoi et al., 2014).

2.2. Reagents and Antibodies

Human Ab serum (14–490E) was obtained from Lonza (Basel, Switzerland). Human macrophage colony-stimulating factor (M-CSF; 300–25), interleukin-10 (IL-10; 571,002), interleukin-4 (IL-4; 574,002), and interferon gamma (IFN-γ; 57,202) were purchased from Peprotech (Rocky Hill, NJ) or BioLegend (San Diego, CA). The sequence of the siRNA against human ATP8B1 (siATP8B1, Cosmo Bio, Tokyo, Japan) was as follows: sense 5′-CAGCCTCTGCTATGTAGA AdTdT-3′ and antisense 5′-TCTACATACGACGGCAdTdT-3′. Negative control siRNA (S20C-0600; Cosmo Bio) was employed as control siRNA (siControl). Antibodies against ATP8B1 were raised in rabbits using an oligopeptide (WIPSESDDKIQHKHRKLKAEQ). The other primary antibodies used are listed in Supporting Table 1. AlexaFluor-labeled secondary antibodies were purchased from Molecular Probes (Life Technologies, Carlsbad, CA). All other chemicals were of analytical grade.

2.3. Preparation of HMDM and their Polarization into Subsets

HPBMo were obtained using a monocyte enrichment cocktail (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions, seeded on Repcell dishes (CellSeed, Tokyo, Japan) for flow cytometric analysis or 12-well plates for the other analyses, and differentiated into HMDM by culturing for 8–11 days in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA) containing 10% human serum, 1% penicillin-streptomycin, and 10 ng/ml human M-CSF (RPMI/M-CSF). Then, the HMDM were either maintained for 6 days in RPMI/M-CSF (nonpolarized HMDM), or cultured in RPMI/M-CSF containing 40 ng/ml human IFN-γ, 20 ng/ml IL-4, or 40 ng/ml IL-10 to elicit polarization into M1, M2a, and M2c subsets, respectively. The cells were cultured at 37 °C in an atmosphere of 5% CO2 in air at 95% humidity. The polarization into M1, M2a, and M2c subsets by the addition of IFN-γ, IL-4, or IL-10, respectively. The polarization of HMDM into M1, M2a, and M2c was confirmed by expression of CD80 (Supporting Fig. 1c), CD23, and CD163 (Fig. 1d), markers of human M1, M2a, and M2c, respectively (Rey-Giraud et al., 2012). Compared with its expression in nonpolarized HMDM, the expression of ATP8B1 was increased by treatment with IL-10 and was abrogated by treatment with IFN-γ and IL-4 (Fig. 1d), indicating that ATP8B1 was exclusively expressed in M2c. qPCR analysis suggested that transcriptional regulation was predominantly responsible for the increase and decrease of ATP8B1 expression in HMDM induced by IL-10 and IL-4 treatment, respectively (Fig. 1e). The expression of ATP8B1 in M2c was confirmed by sorting of nonpolarized and IL-10-treated HMDM based on low and high expression of CD163, a marker of human M2c, and subsequent immunoblot analysis (Fig. 1f). In both types of macrophages, the expression of ATP8B1 was higher in the CD163-positive subset. The lower band in blots for CD163 (Figs. 1c, d, and f) was nonspecific because there was no significant difference in intensity between HMDM with low and high expression of CD163 (Fig. 1f).

3. Results

3.1. Expression of ATP8B1 in M2c

HPBMo were cultured in RPMI/M-CSF to differentiate into HMDM. After 9 days of culture, the cells appeared as a mixture of round and spindle shaped cells, morphologically characteristic of HMDM (Supporting Fig. 1a) (Kittan et al., 2013; Rey-Giraud et al., 2012) that were stained with antibody against 25FS, a human macrophage marker (Pilling et al., 2009), but not with antibody against CD93, a human monocyte marker (Pilling et al., 2009), indicating that the monocytes was correctly differentiated into HMDM (Supporting Fig. 1b). Immunoblot analysis showed that ATP8B1 was expressed on HMDM, but not on granulocytes, lymphocytes, or monocytes (Fig. 1a). By qPCR, ATP8B1 expression appeared after 3 days of culture of HPBMo in RPMI/M-CSF and was maintained in the HMDM (Fig. 1b).

The abundance of ATP8B1 in HMDM varied between individuals (Fig. 1c). HMDM induced by treatment with M-CSF comprise several subsets of macrophages (Chen et al., 2015; Kittan et al., 2013; Rey-Giraud et al., 2012). To identify the subset expressing ATP8B1, HMDM were maintained in RPMI/M-CSF alone or polarized into M1, M2a, and M2c subsets by the addition of IFN-γ, IL-4, or IL-10, respectively. The polarization of HMDM into M1, M2a, and M2c was confirmed by expression of CD80 (Supporting Fig. 1c), CD23, and CD163 (Fig. 1d), markers of human M1, M2a, and M2c, respectively (Rey-Giraud et al., 2012). Compared with its expression in nonpolarized HMDM, the expression of ATP8B1 was increased by treatment with IL-10 and was abrogated by treatment with IFN-γ and IL-4 (Fig. 1d), indicating that ATP8B1 was exclusively expressed in M2c. qPCR analysis suggested that transcriptional regulation was predominantly responsible for the increase and decrease of ATP8B1 expression in HMDM induced by IL-10 and IL-4 treatment, respectively (Fig. 1e). The expression of ATP8B1 in M2c was confirmed by sorting of nonpolarized and IL-10-treated HMDM based on low and high expression of CD163, a marker of human M2c, and subsequent immunoblot analysis (Fig. 1f). In both types of macrophages, the expression of ATP8B1 was higher in the CD163-positive subset. The lower band in blots for CD163 (Figs. 1c, d, and f) was nonspecific because there was no significant difference in intensity between HMDM with low and high expression of CD163 (Fig. 1f).

3.2. ATP8B1-Deficient M2c Show Decreased Expression of M2c Markers and Increased Side Scatter (SSC)

To examine the phenotypic and morphological impact of ATP8B1 in M2c, HMDM were transfected with siControl or siATP8B1 and then cultured in RPMI/M-CSF with or without IL-10 (Fig. 2a, b). No effect of

Fig. 2. Depletion of ATP8B1 decreases expression of M2c markers. HPBMo were seeded, differentiated into HMDM by culture for 8 to 11 days in RPMI/M-CSF, and then transfected with siControl or siATP8B1. Four days later, a second siRNA transfection was performed. During the last five days, the cells were cultured in RPMI/M-CSF with or without IL-10 (40 ng/ml), which induced polarization into M2c. (a) Schematic diagram illustrating the culture procedure. (b) Confirmation of ATP8B1 depletion. The cells were lysed and analyzed by immunoblotting. (c) Lower expression of M2c markers, CD163 and CD14, by ATP8B1 suppression. The cells were stained with fluorochrome-labeled antibodies against CD163, CD14, and CD16, subject to flow cytometry, and the results were analyzed using FlowJo software (v. 10). Expression of each marker in the cells, which was gated according to FSC/SSC properties as described in Supporting Fig. 2a, was determined as MFI (left) and as percentages of CD163+, CD14+, and CD16- cells (right). Data are shown as means ± SEM of triplicate determinations (c), * P < 0.05, ** P < 0.01, *** P < 0.001 versus nonpolarized HMDM; ** P < 0.05, *** P < 0.001 versus siControl. (d, e) Expression of CD163 and CD14 and mean value of SSC in CD16+ cells from IL-10-treated HMDM. FlowJo software (v. 10) was used to gate the CD16+ cells and determine the MFI of CD163 and CD14 (d, left) and the percentages of CD163+ and CD14+ cells (d, right). The CD16+ cells are plotted on a histogram with SSC (e, left) and the mean value of SSC is calculated (e, right). Data shown in (d, e, right) are means ± SEM of triplicate determinations. ** P < 0.01, *** P < 0.001. In (b–e), a representative result of four independent experiments is shown.

2.5. Statistical Analysis

Data are presented as means ± standard error of the mean (SEM), unless otherwise indicated. The differences between two variables and multiple variables were assessed at the 95% confidence level using Student’s t-tests and analysis of variance with a post-hoc Dunnett’s test, respectively. The data were analyzed using Prism software (v. 6; GraphPad Software, La Jolla, CA).
ATP8B1 deficiency on differentiation of HPBMo into HMDM was detected by flow cytometric analysis, which showed that >95% of both siControl- and siATP8B1-transfected cells identified from forward scatter- and SSC-based gating were positive for CD68, a macrophage marker, and negative for CD93, a monocyte marker (Supporting Fig. 2a). The mean fluorescence intensity (MFI) of CD163, CD14, and CD16, surface markers of M2c (Rey-Giraud et al., 2012; Zizzo et al., 2012), and the number of cells positive for each marker were higher in IL-10-treated HMDM than in nonpolarized HMDM (Fig. 2c). Both the MFI and the percent of cells positive for CD163 and CD14, but not for CD16, were markedly inhibited in both IL-10-treated and -untreated HMDM after ATP8B1 depletion (Fig. 2c), which is consistent with the positive correlation between the abundance of ATP8B1 and CD163 in nonpolarized HMDM in individuals (Fig. 1c). The influence

Fig. 3. Influence of ATP8B1 depletion on IL-10/STAT3 signal transduction pathway in HMDM. HMDM deficient for ATP8B1 were prepared as described in Fig. 2. The HMDM were stimulated with or without IL-10 (40 ng/ml) for 60 min and subjected to prepare whole cell lysates (a–d) or RNA (e). (a–c) Tyrosine and serine phosphorylation of STAT3. The prepared cell lysates (5 μg) were analyzed by immunoblotting (a). Expression level of each protein (b) and phosphorylation level of STAT3 (c, d) was quantified as described in the Supporting information. Data shown are means ± SEM of three independent experiments. α, STAT3; β, STAT3; p Y, pS-α; p Y-β, pS-α; p Y-STAT3α; pY, pSTAT3β; p S-α, pSTAT3α; BQL, below the limit of quantitation. (e) mRNA expression of STAT3-regulated genes. The isolated RNA was subjected to qPCR analysis. The expression of SOCS3, SBNO2, and ZNF36 in each reaction was normalized to that of ACTB. Data shown are means ± SEM of triplicate determinations. In (a, e), a representative result of three independent experiments is shown. *, P < 0.05, **, P < 0.01, ***, P < 0.001 versus siControl.
of ATP8B1 on these M2c markers was considered to originate at the mRNA level (Supporting Fig. 3).

Based on these findings, CD16+ cells were gated from IL-10-treated HMDM and used to analyze ATP8B1 function in M2c further. In this subset, 90% of siControl-transfected cells and 65% of siATP8B1-transfected cells were positive for CD163 and CD14 (Fig. 2d, right), indicating that ATP8B1 deficiency prevented excessive tissue disruption because of inflammatory responses, controls inflammatory disease progression, and prevents excessive tissue disruption because of inflammation (Murray, 2006; Ouyang et al., 2011). In HMDM, its anti-inflammatory activity is initiated by binding to the IL-10 receptor (IL-10R) and is predominantly mediated by a transcription factor, signal transducer and activator of transcription 3 (STAT3) (Williams et al., 2004). After the binding of IL-10 to IL-10R, STAT3 is activated via phosphorylation, which causes its translocation from the cytosol to the nucleus, where it binds to the IFN-γ activated sequence in target promoters, and thereby facilitates transcription of anti-inflammatory genes (Murray, 2006, Ouyang et al., 2011).

To understand the mechanism underlying the incomplete IL-10-driven polarization of ATP8B1-deficient HMDM into M2c, the influence of ATP8B1 deficiency on the IL-10/STAT3 signal transduction pathway was explored. IL-10 stimulus elicits phosphorylation of STAT3α, the major isoform of STAT3 in HMDM, at tyrosine 705 and serine 727 and generates two phosphoforms of STAT3α: pY, pS-STAT3α, which is phosphorylated at both tyrosine 705 and serine 727, a canonical activated form of STAT3 (Wen et al., 1995), and pS-STAT3α, phosphorylated only at serine 727, a noncanonical activated form of STAT3 that is required for its optimal activation (Fig. 3a) (Liu et al., 2003; Waitkus et al., 2014; Zhu et al., 2015). Suppression of ATP8B1 in HMDM had no influence on the expression of IL-10R, STAT3α, and STAT3β, an alternative splicing variant of STAT3 that lacks the transactivation domain (Fig. 3a, b). However, ATP8B1 depletion attenuated the IL-10-driven formation of pS-STAT3α, but not of pY, pS-STAT3α (Fig. 3a, c, and d). The amount of pS-STAT3 was 54% lower in the steady state and 80% lower 60 min after IL-10 stimulation in HMDM transfected with siATP8B1 than in those transfected by siControl (Fig. 3d). Consistent with this finding, ATP8B1 suppression markedly reduced the mRNA expression of the STAT3-regulated genes, SOCS3, ZNF36, and SBN02 (El Kasmi et al., 2007; Hutchins et al., 2012; Schaljo et al., 2009), both in the steady state and following IL-10 stimulus (Fig. 3e).

Table 1

| Disease    | Current Age | Gender | Genome | Liver | In vitro Mutagenesis |
|------------|-------------|--------|--------|-------|----------------------|
|            |             |        |        |       | Allele 1 of ATP8B1    |
|            |             |        |        |       | Expression | Localization | Function |
| PFIC1      | no.1        | 3y     | M      | c.2124_2125insGAGCTACAGCTATT GAAGGC (p.R709G) | BQL | + | ND |
| no.2       | 4y          | F      |        | c.316T>C (p.C95R) | BQL | + | ND |
| no.3a      | 10y         | F      |        | c.2941G>A (p.E981K) | NA | ↓ | PM and intracellular |
| no.4       | 10y         | F      |        | c.727del (p.L243fsX28) | NF | + | ND |
| PFIC1-like | no.1b       | 5y     | M      | c.3033-34del (p.L1011fsX18) | BQL | + | ND |
| no.2b      | 10y         | M      |        | c.1585-87del (p.F529del) | NF | + | ND |
| no.3b      | 8y          | M      |        | c.3579_3589del (p.R1193fsX39) | NF | + | ND |
| no.4       | 17y         | M      |        | c.234C>G (p.H78Q) | NF | + | ND |
| PFIC2      | no.1        | 3y     | F      | c.386G>A (p.C129Y) | + | ND |
| no.2       | 2y          | F      |        | c.386G>A (p.C129Y) | + | ND |
| no.3c      | 3y          | F      |        | c.3692G>A (p.R1231Q) | + | ND |
| PFIC2-like | no.1       | 3y     | F      | c.386G>A (p.C129Y) | + | ND |

BQL, below quantification limit; ER, endoplasmic reticulum; F, female; M, male; NA, not available; ND, not done; PM, plasma membrane; +, normal; ↓↓, decreased; ↓↓↓, severely decreased.

3.3. Impaired IL-10/STAT3 Signaling in ATP8B1-Deficient HMDM

IL-10, a potent immunosuppressive cytokine, limits proinflammatory responses, controls inflammatory disease progression, and prevents excessive tissue disruption because of inflammation (Murray, 2006; Ouyang et al., 2011). In HMDM, its anti-inflammatory activity is initiated by binding to the IL-10 receptor (IL-10R) and is predominantly mediated by a transcription factor, signal transducer and activator of transcription 3 (STAT3) (Williams et al., 2004). After the binding of IL-10 to IL-10R, STAT3 is activated via phosphorylation, which causes its translocation from the cytosol to the nucleus, where it binds to the IFN-γ activated sequence in target promoters, and thereby facilitates transcription of anti-inflammatory genes (Murray, 2006, Ouyang et al., 2011).
a. ATP8B1

| No. | Control | PFIC1 | PFIC1-like | PFIC2 | PFIC2-like | Control | PFIC1 |
|-----|---------|-------|------------|-------|------------|---------|-------|
| 1   | 1.2     | 0.7   | 0.75       | 1.3   | 1.3        | 1.5     | 0.55  |
| 2   | BQL     | BQL   | BQL        | BQL   | 2.6        | BQL     | 0.92  |
| 3   | Average: 1.0 | Average: 1.0 | Average: 1.0 | Average: 1.0 | Average: 1.0 | Average: 1.0 | Average: 1.0 |
| 4   | ATP8B1/NaK (a.u.) | ATP8B1/NaK (a.u.) | ATP8B1/NaK (a.u.) | ATP8B1/NaK (a.u.) | ATP8B1/NaK (a.u.) | ATP8B1/NaK (a.u.) | ATP8B1/NaK (a.u.) |
|     | 150     | 100   | 150        | 100   | 150        | 150     | 100   |

b. FLAG-ATP8B1

| Condition | - | WT | Triple (PRC1-like no. 1) | C306R (PRC1 no. 2) | E981K (PRC1 no. 3) |
|-----------|---|----|-------------------------|-------------------|-------------------|
| Cell surface fraction | | | | | |
| Cell lysate | | | | | |
| FLAG (ATP8B1) | | | | | |
| HA (CDC50A) | | | | | |
| NaK | | | | | |
| | | | | | |
| (kDa) | 150 | 75 | 75 | 75 | 75 |
| | 75 | 50 | 50 | 50 | 50 |
| | 100 | 100 | 100 | 100 | 100 |

c. WT | Triple | C306R | E981K

| FLAG (ATP8B1) | NaK | Merge |
|---------------|-----|-------|
| WT | | |
| Triple | | |
| C306R | | |
| E981K | | |

d. EV | ATP8B1+CDC50A

| Time (min) | 0 | 10 | 30 |
|------------|---|----|----|
| NBD-PC (MFI) | | | |
| EV | | | |
| WT | | | |
| Triple (PRC1-like no. 1) | | | |
| C306R (PRC1 no. 2) | | | |
| E981K (PRC1 no. 3) | | | |

e. EV | WT | Triple | C306R | E981K

| NBD-PC (% of EV) | 0 | 25 | 50 | 75 | 100 |
|------------------|---|----|----|----|----|
| EV | | | | | |
| WT | | | | | |
| Triple (PRC1-like no. 1) | | | | | |
| C306R (PRC1 no. 2) | | | | | |
| E981K (PRC1 no. 3) | | | | | |
**ABCB11** (which encodes BSEP) (**PFIC2**) in three patients and in one allele (**PFIC2-like**) in one patient (**Table 1**). The patient with **PFIC1-like** and **PFIC2-like** disease had no mutations in other relevant genes. The genetic diagnosis of **PFIC1** and **PFIC2** was further supported by much lower expression of **ATP8B1** and BSEP in membrane fractions from liver specimens of patients with **PFIC1** and **PFIC2**, respectively, than in control subjects (**Fig. 4a**). Although liver specimens were unavailable for two patients (no.3 and 4) with **PFIC1**, their hepatic expression of **ATP8B1** would be extremely low because they clearly had disease-causing mutations (frameshift mutation and nonsense mutation) or a missense mutation, c.2941G > A (p.E981K) that could decrease exogenous **ATP8B1**-**FLAG** expression by about 80% in CHO-K1 cells expressing HA–**CDC50A**, which forms a complex with **ATP8B1** and assists its correct trafficking to the plasma membrane (**PM**) (**Fig. 4b**) (**Paulusma et al., 2008**).

The abundance of **ATP8B1** in the membrane fractions from liver varied between patients with **PFIC1-like** disease (**Fig. 4a**). Expression of **ATP8B1** was below the quantification limit in **PFIC1-like** patients 1, 2, and 3, but normal in **PFIC1-like** patient 4, who possessed three missense mutations in one allele of **ATP8B1**, each of which has been reported to occur at an allele frequency of about 5% in East Asian populations (http://gnomad.broadinstitute.org/). **ATP8B1** (**FLAG**)–**FLAG**, a mutated form of **ATP8B1**–**FLAG** incorporating these three mutations, showed equivalent cell surface expression and similar cellular localization to **ATP8B1** (**FLAG**)–**FLAG** in CHO-K1 cells (**Fig. 4b, c**). The intrinsic molecular function of **ATP8B1** is to translocate aminophospholipids such as phosphatidylcholine from the outer leaflet to the inner leaflet of biological membranes (**Paulusma et al., 2008; Takatsu et al., 2014**). Incorporation into the inner leaflet of the PM of nitrobenzoxadiazole–labeled phosphatidylcholine (NBD-PC), which cannot be extracted with fatty acid-free BSA, was linear for up to 15 min in **ATP8B1** (**FLAG**)–**FLAG**-transfected CHO-K1 cells (**Fig. 4d**). Its level at 15 min was unaffected by the three mutations harbored by **PFIC1-like** patient 4, but was decreased to an equal degree to that in empty-vector-transfected cells after the introduction of c.916 T > C (p.C306R) and c.2941G > A (p.E981K) mutations (**Fig. 4e**), which were harbored in **PFIC1** patients 2 and 3 (**Table 1**). Overall, these results indicated that **PFIC1-like** patients 1, 2, and 3, but not patients 4, suffered from **PFIC1**.

### 3.5. Diagnosis of PFIC1 in Patients With Normal-GGT PFIC Using Phenotypic Characteristics of M2c

Based on the finding that siRNA-mediated suppression of **ATP8B1** caused incomplete polarization of **HMDM** into M2c (**Fig. 2**), HPBM0 from patients with normal-GGT **PFIC** were differentiated into **HMDM** and their polarization to M2c were facilitated by IL-10 stimulation. Flow cytometric analysis of HPBM0 based on surface expression of CD14 and CD16, which divided HPBM0 into three functionally distinct populations, showed no significant difference between **PFIC1** patients and control subjects (Supporting **Fig. 5**). Correct differentiation of HPBM0 into **HMDM** in the patients with **PFIC1** was confirmed by their staining for CD68, but not for CD93 (Supporting **Fig. 2b**). In all four patients with **PFIC1**, IL-10-treated **HMDM** showed decreased expression of CD163 and CD14 and increased SSC (**Fig. 5a**), compared with cells from control subjects, which were prepared from the HPBM0 of at least three individuals to minimize interindividual variability (**Fig. 1c**). The IL-10-treated **HMDM** from patients with **PFIC2** and **PFIC2-like** disease, who in the early stages of disease present with clinical symptom similar to those of **PFIC1** (**Davit-Spraul et al., 2009**), showed an almost identical phenotypic and morphological pattern to those of the control subjects. The MFI of CD163 and CD14 and the mean value of SSC in IL-10-treated **HMDM** calculated relative to the values in control subjects, were 61 ± 8% (P < 0.05) and 68 ± 6% (P < 0.01) lower and 57 ± 8% (P < 0.01) higher, respectively, in patients with **PFIC1** than in patients with **PFIC2** and **PFIC2-like** disease (**Fig. 5b**). The IL-10-treated **HMDM** from **PFIC1-like** patients 1, 2, and 3, but not patient 4, showed the same phenotypic and morphological pattern as those from the patients with **PFIC1** (**Fig. 5c, d**), indicating that the same diagnosis could be made using M2c as was made based on the amount of **ATP8B1** in liver and the in vitro mutually recognition study.

### 4. Discussion

**PFIC1** shares many clinical and liver pathological features with other subtypes of normal-GGT **PFIC**, but differs in the available therapeutic options (**Davit-Spraul et al., 2009**). Patients with **PFIC1** develop graft steatosis with or without fibrosis as well as severe diarrhea after LTx and sometimes need re-transplantation (Hori et al., 2011; Miyagawa-Hayashino et al., 2009), whereas it is the only curative option for the disease-related liver cirrhosis in individuals with other subtypes of normal-GGT **PFIC**. Therefore, to identify the preferred first-line therapy for these patients accurately, it is of the highest priority to discriminate **PFIC1** correctly from other types of normal-GGT **PFIC** at an early phase of the disease.

The current study showed using siRNA-mediated **ATP8B1** suppression that **ATP8B1** contributes to IL-10-driven polarization of **HMDM** into M2c, and explored the usefulness of this finding for the diagnosis of **PFIC1** using **HMDM** from patients with **PFIC1**, **PFIC2**, and undiagnosed normal-GGT **PFIC**. This method resulted in the correct diagnosis of all the tested patients with **PFIC1** who had disease-causing mutations in both alleles of **ATP8B1** (**Fig. 4** and **Table 1**), because IL-10-treated **HMDM** from patients with **PFIC1** showed significantly lower expression of typical M2c surface markers and higher SSC than those from control subjects. **HMDM** from patients with **PFIC2** and **PFIC2-like** disease, other subtypes of normal-GGT **PFIC**, showed an almost identical phenotypic and morphological pattern to controls (**Fig. 5a, b**).

The results of this analysis in four patients with **PFIC1-like** disease who harbored only one mutant allele of **ATP8B1** and no mutations in the other genes responsible for neonatal/infantile intrahepatic cholestasis, meant that three of four patients (patients 1 to 3) were diagnosed with **PFIC1** (**Fig. 5c, d**). These three patients, who showed no **ATP8B1** expression in liver specimens (**Fig. 4a**), presented with the typical clinical symptoms of **PFIC1**, including cholestasis with normal GGT, intractable itching, diarrhea/steatorrhea, failure to thrive, and mild intellectual disability. The histology of their liver specimens showed mild fibrosis with hepatocellular and canalicular cholestasis lacking giant cell transformation, which is compatible with **PFIC1** (**Table 2**) (**Davit-Spraul et al., 2010; Pawlikowska et al., 2010; Suchy et al., 2014**). Furthermore, an electron

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**Fig. 4.** Diagnosis of PFIC subtype using liver specimens and in vitro mutagenesis study in the patients with normal-GGT PFIC. (a) Expression of **ATP8B1** and **BSEP** in livers of patients with normal-GGT PFIC. Membrane fractions were prepared from liver specimens of patients and 5 μg (left panel) and 20 μg (right panel) of the specimens were subjected to immunoblotting. The signal intensity of **ATP8B1** and **BSEP** relative to that of NaK is presented below each panel. Control 1: HBV, Control 2; HCV, Control 3; HCV, Control 4; Alagille syndrome. a.u., arbitrary unit; BQL, below the limit of quantitation; NaK, Na+ K+ ATPase-ε1 subunit. (b-d) Characterization of missense mutations of **ATP8B1** in patients with **PFIC1** and **PFIC1-like** disease. CHO-K1 cells were transfected with pShuttle-**ATP8B1** (**FLAG**), **ATP8B1** (**FLAG**), or **ATP8B1** (**FLAG**) together with pShuttle-HA–**CDC50A** and subjected to cell surface biotinylation (b), immunocytochemistry (c), and a fluorescence assay (d, e). CHO-K1 cells transfected with the corresponding empty vector were employed as control cells. A representative result of at least two independent experiments is shown. (b) Protein expression. Upper, the cells were biotinylated, lysed, precipitated with streptavidin, and then analyzed by immunoblotting. Lower, quantification of the amount of the mutated **ATP8B1** in the cell surface fraction. The band intensity shown in the upper panel was quantified as described in the Supporting information. Each bar represents the mean ± SEM of triplicate determinations. *P < 0.001. BQL, below the limit of quantitation; EV, empty vector; WT, wild type.
microscopic analysis showed Byler's bile in bile canalicus of these patients, which is highly suggestive of PFIC1 (Suchy et al., 2014). The other disease-causing mutation in ATP8B1 present in these patients may be outside the sequenced regions in areas such as transcriptional regulatory elements or untranslated regions. The other patient with PFIC-like disease (patient 4) showed similar liver histology to that of PFIC1 and presented with clinical symptoms similar to those of PFIC1 since 6 months old, including cholestasis with normal GGT, failure to
thrive, severe pruritus, severe intellectual disability, and sensorineural deafness (Table 2) (Davit-Spraul et al., 2010, Pawlikowska et al., 2010, Suchy et al., 2014). However, in contrast to the situation with PFIC1, his serum bilirubin level returned to normal at 19 months old and his serum transaminases were elevated only episodically. These clinical symptoms that differed from those of PFIC1 were consistent with the normal ATP8B1 expression in his liver specimen (Fig. 4a), and the lack of a significant impact of the three mutations he possessed on the expression, cellular localization, and transport activity of ATP8B1 (Fig. 4b–d). Genetic diagnosis of this patient by whole-exome sequencing is under way. Together, these results demonstrated that the phenotypic and morphological analysis of IL-10-treated HMDM made it possible to assess the functional deficiency of ATP8B1 in individuals with a clinical diagnosis of normal-GGT PFIC.

II-10/STAT3 signaling is initiated by binding of IL-10 to the IL-10R and subsequent tyrosine phosphorylation of the IL-10R by Janus kinase 1. This in turn results in STAT3, an obligate transcription factor for IL-10 signaling, docking with IL-10R and becoming activated by phosphorylation (Williams et al., 2004). A functional deficiency of ATP8B1 in HMDM attenuated the IL-10/STAT3 signal transduction pathway by decreasing pS-STAT3 (Fig. 3), which is required for its optimal activation (Liu et al., 2003; Waikutus et al., 2014; Zhu et al., 2015), and resulted in the incomplete IL-10-driven polarization of HMDM into M2c (Figs. 2 and 5). ATP8B1, a member of the P4 subfamily of P-type adenosine triphosphatases, is expressed on the PM and translocates aminophospholipids from the outer leaflet to the inner leaflet, thereby contributing to making the PM a rigid, liquid-ordered membrane (Paulusma et al., 2008; Takatsu et al., 2014). Although the mechanism underlying the regulation of pS-STAT3 by ATP8B1 is unclear, a functional deficiency of ATP8B1 may disrupt the well-organized aminophospholipid asymmetry of the PM (Paulusma et al., 2009) and affect tethering to the PM of the kinases responsible for generation of pS-STAT3. This concept is supported by the observation that ATP8B1 depletion inactivates protein kinase Cζ, whose activation depends on recruitment to the PM (Frankenberg et al., 2008). Various serine kinases, including JNK, ERK, mTOR, and several forms of protein kinase C, are implicated in the phosphorylation of STAT3 at serine 727, but their contribution differs in each cell type and with different stimuli (Aziz et al., 2007; Decker and Kovarik, 2000). Identification of the kinases that mediate formation of pS-STAT3 in HMDM stimulated with IL-10 will clarify the molecular mechanism underpinning the IL-10-driven polarization of HMDM into M2c and the molecular role of ATP8B1 in this process.

Treatment of HMDM with IL-10 to elicit their polarization into M2c, suppresses fluid phase pinocytosis and mannose receptor-mediated uptake (Montaner et al., 1999) and induces Mer tyrosine kinase, a member of the TAM subfamily of receptors that recognizes phosphatidylserine through protein S and Gas6. This enables HMDM to clear preferentially and efficiently early apoptotic cells derived from various processes including normal homeostasis, tissue turnover, and immune responses against pathogens (Xu et al., 2006; Zizzo et al., 2012). Therefore, M2c suppress persistent apoptosis and the accumulation of secondary necrotic cells, which are highly inflammatory because their autolysis releases cytotoxic, proinflammatory and immunogenic molecules including damage-associated molecular patterns (Silva, 2010). The anti-inflammatory actions of M2c are amplified and prolonged by their uptake of apoptotic cells and subsequent IL-10 secretion, generating a positive feedback loop of M2c homeostasis (Xu et al., 2006, Zizzo et al., 2012). ATP8B1 deficiency in HMDM resulted in incomplete polarization of HMDM into M2c (Figs. 2, 3, and 5), which might prevent the resolution of inflammation and contribute, at least in part, to the pathogenesis of the clinical features of PFIC1, including steatohepatitis following LTX (Hori et al., 2011; Miyagawa-Hayashino et al., 2009), pancreatitis (Pawlikowska et al., 2010), and atherosclerosis (Nagasaka et al., 2005), in which cholesterol-laden apoptotic foam cells progress to secondary necrosis, causing inflammation and plaque instability. This could be supported by a recent study using THP-1-derived Mc, which found that ATP8B1 depletion prevents LPS-induced internalization of TLR4 and may thereby induce a chronic inflammatory response (van der Mark et al., 2017). However, the pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes generated in HMDM in response to pathophysiologic stimuli are distinct from THP-1-derived Mcs in which no induction of cell surface markers characteristic of M2a and M2c by stimulation with IL-4 or IL-10, respectively, was observed (Shiratori et al., 2017). Further studies to clarify the roles of ATP8B1 in M2c and the functions of M2c in liver physiology and pathophysiology should pave the way to understanding the pathogenic mechanism of progressive cholestatic liver failure and the extrahepatic manifestations of PFIC1, and allow the development of new therapies for PFIC1.

In conclusion, our present study showed that ATP8B1 deficiency caused incomplete polarization of HMDM into M2c via impairment of the IL-10/STAT3 signal transduction pathway. Genome sequencing and liver histological analysis, both of which are currently employed to diagnose PFIC1, are insufficient for that purpose because of the shared features of liver histology and the difficulties in identifying disease-causing mutations. Phenotypic and morphological analyses of IL-10-driven HMDM help to discriminate PFIC1 patients from individuals who have a clinical diagnosis of normal-GGT PFIC and no apparent disease-causing mutations in ATP8B1. The application of these findings as an alternative diagnostic method for PFIC1 must be validated by future studies including larger numbers of patients with neonatal/infantile cholestasis than was possible in this study. If confirmed, this method may assist with identifying the optimal treatment plan in patients with normal-GGT PFIC and in understanding the actual prevalence and clinical course of PFIC1.

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Table 2
Clinical and biochemical features and liver histology in PFIC1 and PFIC1-like patients.

| Features                                      | PFIC1 diagnosis of PFIC1 [% in PFIC1] | PFIC1-like diagnosis of PFIC1 [% in PFIC1-like] |
|-----------------------------------------------|-------------------------------------|-----------------------------------------------|
| Presenting symptoms                           |                                     |                                               |
| Age of onset                                  | Before 1 year                       | 2 months                                      |
| Cholestasis                                   | + [100%]                            | +                                             |
| Jaundice                                      | + [100%]                            | +                                             |
| Intractable itching                           | + [100%]                            | +                                             |
| Diarrhea                                      | + [61%]                             | +                                             |
| Steatorrhea                                    | + [NA]                              | +                                             |
| Sensorineural deafness                        | + [31%]                             | +                                             |
| Failure to thrive                             | + [90%]                             | +                                             |
| Short stature                                  | + [NA]                              | +                                             |
| Weight-for-height                             | >Normal [NA]                        | >Normal                                        |
| Mental retardiation                           | + [NA]                              | +                                             |
| Pneumonia                                     | + [13%]                             | +                                             |
| Rickets                                       | + [46%]                             | +                                             |
| Pancreatitis                                  | + [12%]                             | +                                             |
| Serum AST, ALT                                | <2 × Normal [NA]                    | <2 × Normal                                   |
| GGT                                           | Low to normal [NA]                  | Low to normal                                 |
| Total bilirubin                               | Elevated [NA]                       | Elevated                                      |
| Total bile acid                               | Elevated [NA]                       | Elevated                                      |
| Liver histology                               | + [NA]                              | +                                             |
| Giant transformation of hepatocytes           | No or few [NA]                      | +                                             |
| Fibrosis                                      | + [NA]                              | +                                             |
| Surgical procedure (age)                      | PEBD (4 months)                     | PEBD (4 months)                               |
| LDLTx (2 years 4 months)                      |                                   | LDLTx (3 years 6 months)                      |
| LDLTx (2 years 4 months)                      |                                   | PEBD (1 years 3 months)                       |

LDLTx, living donor liver transplantation; NA, not available; PEBD, percutaneous external biliary drainage; PBD, partial internal biliary diversion.

Conflict of Interest
The authors declare no conflicts of interest associated with this study.

Author Contributions
H.H. conceived the study, designed and performed the experiments, collected and analyzed the data, and wrote the manuscript. S.N. designed and performed the experiments, collected and analyzed the data. Y.H. performed in vitro mutagenesis study and analyzed the data. T.T. performed targeted next-generation sequencing and analyzed the data. Y.H., H.K., M.S., K.M., S.W., D.A., S.N., K.M., A.F., M.K., and A.I. provided biological samples and clinical information of PFIC patients. M.S. provided biological samples and clinical information of PFIC patients and revised the manuscript for intellectual content. H.N. K.B., and H.K. revised the manuscript for intellectual content. All authors approved the manuscript before submission.

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Appendix A. Supplementary data
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