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Bile Salt Export Pump-Reactive Antibodies Form a Polyclonal, Multi-Inhibitory Response in Antibody-Induced Bile Salt Export Pump Deficiency

Jan Stindt,[1]* Stefanie Kluge,[1]* Carola Dröge,[1] Verena Keitel,[1] Claudia Stross,[1] Ulrich Baumann,[2] Florian Brinkert,[3] Anil Dhawan,[4] Guido Engelmann,[5,6] Rainer Ganschow,[3,7] Patrick Gerner,[8,9] Enke Grabhorn,[3] A. S. Knisely,[10]** Khalid A. Noli,[11] Ieva Pukite,[12] Ross W. Shepherd,[13] Takehisa Ueno,[14] Lutz Schmitt,[15] Constanze Wiek,[16] Helmut Hanenberg,[16] Dieter Häussinger,[1] and Ralf Kubitz[1,17]

Progressive familial intrahepatic cholestasis type 2 (PFIC-2) is caused by mutations in *ABCB11*, encoding the bile salt export pump (BSEP). In 2009, we described a child with PFIC-2 who developed PFIC-like symptoms after orthotopic liver transplantation (OLT). BSEP-reactive antibodies were demonstrated to account for disease recurrence. Here, we characterize the nature of this antibody response in 7 more patients with antibody-induced BSEP deficiency (AIBD). Gene sequencing and immunostaining of native liver biopsies indicated absent or strongly reduced BSEP expression in all 7 PFIC-2 patients who suffered from phenotypic disease recurrence post-OLT. Immunofluorescence, western blotting analysis, and transepithelial transport assays demonstrated immunoglobulin (Ig) G-class BSEP-reactive antibodies in these patients. In all cases, the N-terminal half of BSEP was recognized, with reaction against its first extracellular loop (ECL1) in six sera. In five, antibodies reactive against the C-terminal half also were found. Only the sera recognizing ECL1 showed inhibition of transepithelial taurocholate transport. In a vesicle-based functional assay, transport inhibition by anti-BSEP antibodies binding from the cytosolic side was functionally proven as well. Within 2 hours of perfusion with antibodies purified from 1 patient, rat liver showed canalicular IgG staining that was absent after perfusion with control IgG. *Conclusions:* PFIC-2 patients carrying severe BSEP mutations are at risk of developing BSEP antibodies post-OLT. The antibody response is polyclonal, targeting both extra- and intracellular BSEP domains. ECL1, a unique domain of BSEP, likely is a critical target involved in transport inhibition as demonstrated in several patients with AIBD manifest as cholestasis. (HEPATOLOGY 2016;63:524-537)

Progressive familial intrahepatic cholestasis (PFIC) is a group of inherited cholestatic diseases of hepatocellular origin often starting in early infancy.[1,2] PFIC has an estimated incidence of 1-2 per 100,000 births[3] and can be divided into three subtypes with differences in clinical, biochemical, and histological features (PFIC-1-3). PFIC-1, also termed Byler's disease, is characterized by normal gamma-glutamyl

Abbreviations: ABCB11, adenosine triphosphate binding cassette transporter superfamily, subfamily B, member 11; AIBD, antibody-induced BSEP deficiency; ATP, adenosine triphosphate; BSEP, bile salt export pump; CsA, cyclosporin A; Cy3, cyanine 3; ECL1, first extracellular loop of BSEP; EYFP, enhanced yellow fluorescent protein; FIC1, familial intrahepatic cholestasis 1; GGT, gamma-glutamyl transpeptidase; Ig, immunoglobulin; MDR, multidrug resistance; mRNA, messenger RNA; mTECs, medullary thymic epithelial cells; NTCP, Na^+/taurocholate cotransporting polypeptide; OLT, orthotopic liver transplantation; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PFIC, progressive familial intrahepatic cholestasis; SDS, sodium dodecyl sulfate; TC, taurocholate; TCR, T-cell receptor; TSAs, tissue-specific antigens; WT, wild type.

From the [1]Department of Gastroenterology, Hepatology and Infectious Diseases, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany; [2]Pediatric Gastroenterology and Hepatology, Department for Pediatric Kidney, Liver and Metabolic Diseases, Hannover Medical School, Hannover, Germany; [3]Pediatric Hepatology and Liver Transplantation, Transplantation Center, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [4]Pediatric Liver Center, King's College Hospital, London, United Kingdom; [5]Department of General Pediatrics, Heidelberg University Hospital, Heidelberg, Germany; [6]Pediatric Clinic, Lukaskrankenhaus GmbH, Neuss, Germany; [7]Clinic of General Pediatrics, University Hospital, Bonn, Germany; [8]Department for Pediatric Nephrology, Gastroenterology, Endocrinology and Transplant Medicine, Clinic for Pediatrics II, University Children's Hospital Essen, University Duisburg-Essen, Essen, Germany; [9]Children's Hospital, Albert Ludwigs University, Freiburg, Germany; [10]Institute of Liver Studies, King's College Hospital, London, United Kingdom; [11]Clinical Laboratory Services Division, Dhahran Health Center, Dhahran, Saudi Arabia; [12]Latvian Center of Pediatric Gastroenterology/Hepatology, University Children's Hospital, Riga, Latvia; [13]Texas Children's Hospital Liver Center, Baylor College of Medicine, Gastroenterology, Houston, TX; [14]Pediatric Surgery/Pediatric Liver and GI Transplant, Osaka University Graduate School of Medicine, Osaka, Japan; [15]Institute of Biochemistry, Heinrich Heine University, Düsseldorf, Germany; [16]Department of Otorhinolaryngology (ENT), Heinrich Heine University School of Medicine, Düsseldorf, Germany; and [17]Medical Clinic I, Bethanien Hospital, Moers, Germany.

transpeptidase (GGT) levels and is associated with extrahepatic features such as pancreatitis, diarrhea, and hearing impairment. It is caused by mutations in the *ATP8B1* gene[4] encoding familial intrahepatic cholestasis 1 (FIC1), an aminophospholipid flippase. PFIC-2, like PFIC-1, is associated with low GGT levels and caused by mutations in the bile salt export pump (BSEP) expressed by the *ABCB11 (*adenosine triphosphate binding cassette transporter, subfamily B, member 11) gene. PFIC-2 is also referred to as BSEP deficiency.[5] PFIC-3 is caused by mutations in the *ABCB4* gene, which encodes the multidrug resistance protein 3, a phosphatidylcholine (PC) floppase.[6,7] When PC is reduced in bile, formation of mixed micelles is disturbed.[8] The resulting increase in free bile salts leads to injury of the biliary tree, resulting in elevated GGT levels.[7]

BSEP is exclusively expressed in the microvillar domains of the canalicular hepatocyte membrane[9]; it is the principal transporter of bile salts from hepatocytes into bile.[9,10] Missense, nonsense, indel, or splice-site mutations of *ABCB11* may impair bile salt transport as a result of reduced or absent protein expression, altered splicing, disturbed protein processing and trafficking, or reduced transmembrane transport.[10] Milder forms of BSEP deficiency are termed, depending on the clinical setting, benign recurrent intrahepatic cholestasis type 2, intrahepatic cholestasis of pregnancy, and drug-induced liver injury.[5]

PFIC-2 manifests in infancy with jaundice, severe intractable pruritus, diarrhea, complications of fat-soluble vitamin deficiency, and growth failure.[11] Serum alanine aminotransferase activity and bile salt levels are high; GGT levels are lower than expected for the degree of conjugated hyperbilirubinemia. Histological features of PFIC-2 at presentation are lobular and portal fibrosis and inflammation, with intralobular cholestasis accompanying hepatocellular disarray, giant-cell change, and necrosis, ascribed to retention of bile salts within hepatocytes.[3,12] In many patients, BSEP expression is absent or reduced on immunohistochemical or immunofluorescent study of liver tissue.[13-15] Without treatment, PFIC-2 progresses to end-stage liver disease, sometimes with hepatobiliary malignancy,[15,16] leading to death in childhood.[17] Only orthotopic liver transplantation (OLT) yields good outcome in most PFIC-2 patients.[5,11]

In some patients with PFIC-2, a phenotype suggesting recurrence of BSEP deficiency develops post-OLT. In 2009, we described a girl with PFIC-2 caused by lack of BSEP expression resulting from three homozygous nucleotide substitutions in *ABCB11*. After OLT, her serum contained high-affinity antibodies directed against the first extracellular loop (ECL1) of BSEP, with strong canalicular immunoglobulin (Ig) G deposits in two consecutive liver allografts.[18] We and others proposed that BSEP-reactive antibodies cause cholestasis by inhibiting BSEP transport activity.[18,19] Since then, only a few further cases of antibody-induced BSEP deficiency (AIBD) have been reported.[19-22] We here present a detailed analysis of the BSEP-reactive antibody repertoire causing AIBD, and of these antibodies' functional effects, in sera from 7 more AIBD patients.

Patients and Methods

Additional method descriptions can be found in the Supporting Information.

Patients. Charts of all patients in this study were reviewed. Table 1 summarizes patient details, genetic information, course of disease recurrence, and treatment regimens. Serum samples for antibody detection were stored at -20°C. A liver sample from a noncholestatic patient undergoing partial hepatectomy because of liver metastasis of a colon carcinoma was used for detecting antibodies in patients' serum with canalicular reactivity. Serum of a healthy 35-year-old man was used as a non-OLT control throughout this study. All patients or their parents gave written consent for investigation. This

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**These authors contributed equally.*

***Present address: Institut für Pathologie, Medizinische Universität Graz, Auenbruggerplatz 25, A-8036, Graz, Österreich/Austria.*

Address reprint requests to: Verena Keitel, M.D., or Ralf Kubitz, M.D., Department of Gastroenterology, Hepatology and Infectious Diseases, Heinrich Heine University, Düsseldorf, Moorenstrasse 5, D-40225 Düsseldorf, Germany. Email: Verena.Keitel@med.uni-duesseldorf.de; fax: +49 211 8104847.

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Table 1. Data of AIBD and Non-AIBD Control Patients

Patient	1	2	3	4	5	6	7	Control (8)	Control (9)
AIBD	Yes	Yes	Yes	Yes	Yes	Yes	Yes	PFIC-2 non-AIBD	non-PFIC-2 non-AIBD
YOB, sex Born in	2002, male Germany	2004, male Saudi Arabia	2002, female USA	2009, male Lithuania	2008, male Russia	2007, male Japan	1999, female Saudi Arabia	2009, male Germany	1996, male Germany
BSEP mutations (protein effect)	c.150+3A>C (aberrant splicing) c.2783_2787dup5 (p.K930Efs79X)	c.2371C>T (p.Q791X)	c.1723C>T (p.R575X) [2] c.2178+1G>T (aberrant splicing)	c.2926C>T (p.Q976X)	Coding exons 6-9 missing	c.953-954delAA (p.K318Rfs33X) c.1425T>A (p.C475X)	c.3213+1delG (p.D1072Tfs25X)	c.1259T>A (p.I420N)c.1445A>G (p.D482G)	None
Zygosity	comp het[1]	homozygous	comp het	homozygous	homozygous	comp het	homozygous	comp het	
BSEP expression before OLT	Reduced	Likely absent*	Absent	Absent	Absent	Absent	Absent	Likely present*	Normal
Age at OLT, years	3.1	1.9	1.6	1.4	1.3	1.9	3.1	1.6	3.3
Recurrence after OLT, months	45	37	3	9	42	20	108	2	24
Symptoms and signs at time of recurrence	Diarrhea, pruritus	Diarrhea, poor weight gain, progressive jaundice/ pruritus	Pruritus, diarrhea, abdominal pain, vomitus, fatigue, fever	Jaundice, pruritus, ↑transaminases	Jaundice, pruritus, ↑INR, poor weight gain	Jaundice	Jaundice, pruritus	Vomiting, dehydration. diarrhea ↑transaminases	Recurrent acute rejection ↑transaminases
GGT at time of recurrence, U/L	16	43	Not available	36	31	108	10	86	Not available
Potential AIBD triggering factors	None identified	Episode 1: Tac dose↓, *de novo* HBV infection Episode 2: change of MMF formulation	High doses of antibiotics (Cotrim, azithromycin, amoxicillin)	Low EBV titer w/o clinical symptoms, fluctuation in CsA level, acute rejection with toxic injury	Fluctuation in CsA level, mild rejection	Immunoadsorption + Tac + steroid↓	None identified	–	–
Initial IS	Basiliximab + CsA + steroid	Tac + steroid	Tac + steroid	Basiliximab	Basiliximab + CsA + steroid	Tac + steroid	Tac + steroid	CsA	CsA
IS at time of recurrence	Tac	↑Tac dose + steroid	Tac + steroid	CsA + steroid	CsA	Tac + steroid	Tac	Tac	CsA
IS for treatment of recurrence	Trim	MMF + Tac	↑Tac dose + steroid	Tac + azathioprin	CsA + everolimus	Tac + steroid + mizoribine	steroids + MMF + Tac	Tac	CsA + steroid (later + Tac)
Additional treatment	Immunoadsorption + rituximab	Entecavir	Metronidazol	Steroid pulse + immunoadsorption, plasmapheresis + rituximab	Immunoadsorption, plasmapheresis + rituximab	Steroid pulse + plasmapheresis + rituximab	–	–	Plasmapheresis
IgG deposits in transplant biopsies	Yes	None detected	Yes	Yes	Yes	Not available	Yes	None detected	None detected
Maximum anti-canalicular antibody titer	1:16,000	1:1,000	1:10,000	1:8,000	1:8,000	1:16,000	1:6,400	1:100	1:50

Abbreviations: comp het, compound heterozygous; Cotrim, trimethoprim/sulfamethoxazole; CsA, cyclosporin A; EBV, Epstein-Barr virus; HBV. Hepatitis B virus; INR, international normalized ratio; IS, immunosuppression; MMF, mycophenolate-mofetil; Tac, tacrolimus; Trim, trimethoprim; YOB, year of birth.

*Native liver biopsies were not examined for BSEP expression.

study was performed according to the guidelines of the declaration of Helsinki.

ABCB11 ***Sequencing From Genomic DNA.*** Genomic DNA was extracted using the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). All 27 coding exons of *ABCB11*, with exon-intron transitions, were amplified and sequenced as previously described.[23] Data were compared with reference sequence NM_003742.2 (Gene ID: 8647); variations were verified by resequencing and designated according to the Human Genome Variation Society guidelines.[24]

Immunofluorescence Staining of Liver Cryosections. As previously described,[14,23] sera diluted up to 1:16,000 were tested for immunoreactivity using methanol-fixed cryosections of noncholestatic human liver. A murine monoclonal antibody against multidrug resistance-associated protein 2 (M2I-4; Alexis, Grünberg, Germany) served as a canalicular marker (used at 1:25). Goat anti-human IgG/cyanine 3 (Cy3; Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-mouse IgG/Alexa Fluor 488 (Invitrogen, Karlsruhe, Germany) were used as secondary antibodies (each at 1:500). For detection of canalicular IgG deposits, fixed cryosections of patient transplant liver biopsies and perfused rat liver were immunostained as described above. BSEP expression in native liver of patient 1 was analyzed using the K24 antiserum raised against the C-terminus of human BSEP.[25] Specimens were analyzed with a LSM 510 Meta confocal laser scanning microscope (Zeiss, Jena, Germany).

Analysis of Patient Sera for anti-BSEP Reactivity in Transiently Transfected HEK293 Cells. HEK293 (human embryonic kidney) cells were transiently transfected with wild type (WT) BSEP-EYFP (enhanced yellow fluorescent protein), BSEP-1-659-EYFP, or BSEP-659-1321-EYFP[18] using X-treme Gene HP (Roche Applied Science, Mannheim, Germany). After 48 hours, cells were fixed with ice-cold methanol for 30 seconds and stained with human sera (1:50) for 1 hour using goat anti-human IgG/Cy3 as the secondary antibody (1:500). The monoclonal anti-BSEP antibody, F-6 (Santa Cruz Biotechnology, Heidelberg, Germany), served as a positive staining control (1:100), with goat anti-mouse IgG/Cy3 as secondary antibody (1:500). Cell nuclei were stained with Hoechst 34580 or 4′,6-diamidino-2-phenylindole (Invitrogen). Cells were visualized by confocal laser-scanning microscopy.

Cloning, Expression, and Purification of the First Extracellular Loop of Human BSEP. The first extracellular loop of human BSEP, with an N-terminal hexahistidine tag for purification, was expressed as a C-terminal EYFP fusion protein (ECL1-EYFP) in *Escherichia coli* (see Supporting Information for details of cloning strategy, expression, and purification).

Analysis of Patient Sera for Anti-BSEP Reactivity by Western Blotting. Ten micrograms of *Sf*9 membrane vesicles (either empty control vesicles or vesicles containing human BSEP; Solvo Biotechnology, Budapest, Hungary) or 0.5 μg of purified ECL1-EYFP were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. For detection of BSEP-reactive IgG, membranes were first incubated for 1 hour with patient or control serum (1:100) followed by goat anti-human IgG/horseradish peroxidase (1:10,000) as the secondary antibody. The anti-BSEP antibody F-6 was used at 1:1,000 as a positive control.

Transepithelial Transport Assays. Generation of LLC-PK_1 cells stably expressing BSEP and/or NTCP (the Na^+/taurocholate cotransporting polypeptide, here facilitating cellular uptake of taurocholate [TC]) is described in the Supporting Information. For transepithelial transport studies, cells were seeded at high density onto ThinCert™ cell-culture inserts (0.4-μm pore size; Greiner Bio One, Frickenhausen, Germany) and cultured for 2 days. Cell monolayers were routinely used at a net transepithelial electric resistance of at least 500 Ωcm^2. To compensate for the lack of CO_2-enriched atmosphere during the transport inhibition studies, both apical and basal medium were exchanged for 1 and 2 mL, respectively, of 25 mM of HEPES buffered medium (pH, 7.5). Then, 100 μL of apical medium were replaced by either patient or control serum (thus diluted 1:10) for overnight incubation of the apical face of the cell monolayer. Alternatively, 4 μg of F-6 antibody, which does not recognize ECL1, was used as a negative control. A mixture of 1 mM of unlabeled sodium TC (Sigma-Aldrich, St. Louis, MO) and 200 μM of [^{3}H]-TC (PerkinElmer, Waltham, MA) at a ratio of 1:100 was added to the basal medium to a final concentration of 5 μM, and cells were incubated for 1 hour at 37°C. Then, cell-culture inserts were removed, apical and basal media were collected, and inserts were gently washed three times on their apical and basal sides with 1 and 2 mL of ice-cold phosphate-buffered saline (PBS), respectively. Finally, the cell monolayer was lysed in 500 μL of 0.5 M of NaOH with 0.1% (w/v) SDS. Then, 250 μL of each cell lysate (or 500 μL of medium) was mixed with 4 mL of liquid scintillation fluid (Ultima Gold; BD Biosciences, Heidelberg, Germany) for 20 seconds before measurement in a scintillation counter (Packard Instruments, Frankfurt, Germany).

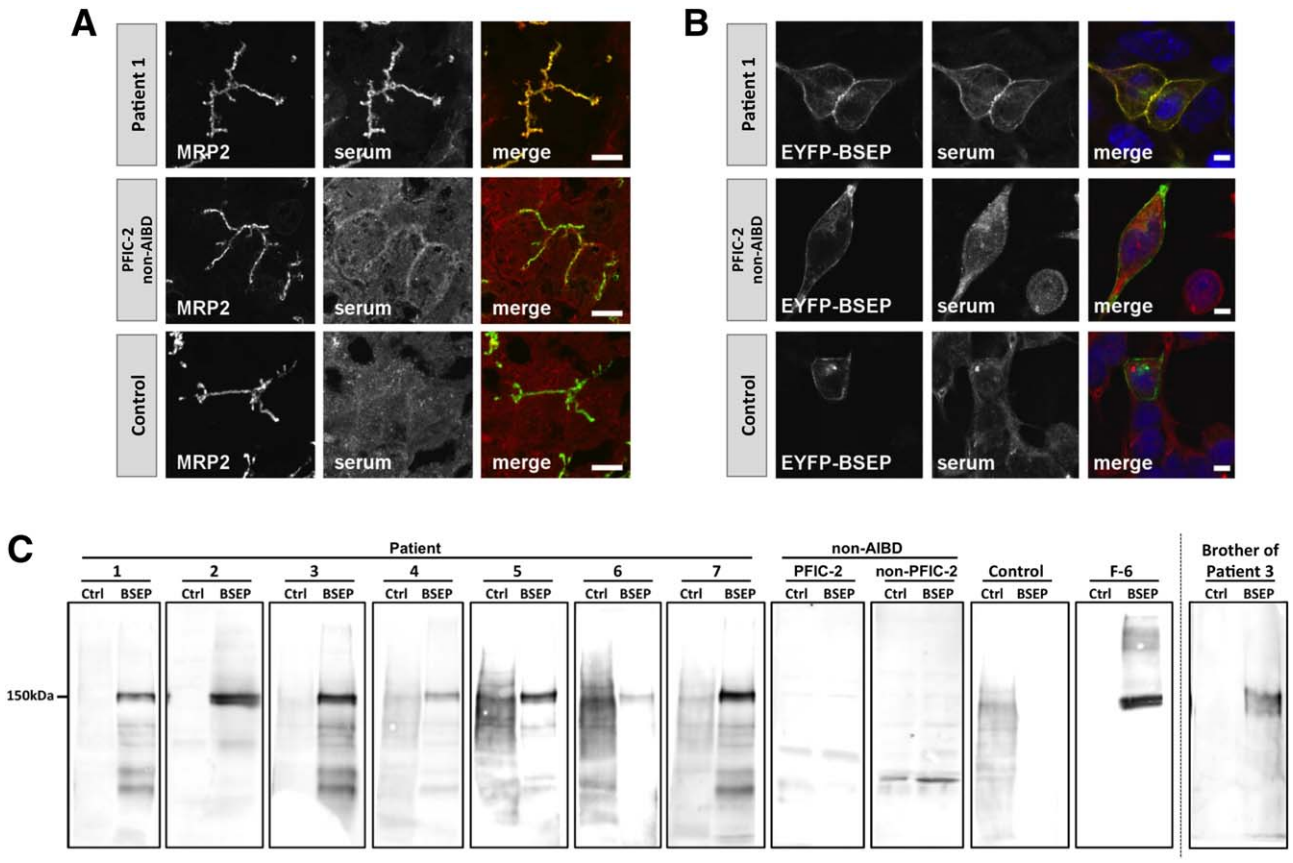

Fig. 1. AIBD sera specifically recognize human BSEP. (A) Post-OLT sera from AIBD patient 1 and a non-AIBD, PFIC-2 control yield canalicular staining patterns in cryosections of healthy human liver whereas naïve, non-OLT control serum does not. MRP2 was immunostained as a canalicular marker. The non-AIBD control sera only stain up to dilutions of 1:100, whereas all AIBD sera stain at maximal dilutions ranging from 1:1,000 up to 1:16,000. (B) All AIBD sera recognize human full-length BSEP-EYFP expressed in HEK293 cells (patient serum 1 shown). In contrast, non-AIBD sera do not recognize BSEP (non-AIBD PFIC-2 control shown). Bars = 10 μm. (C) Likewise, only the seven AIBD sera detect recombinant human BSEP (expressed in *Sf*9 cells) on nitrocellulose membranes (Ctrl, empty control vesicles; BSEP, vesicles containing human BSEP). The non-AIBD, PFIC-2 control likely had some BSEP expression before OLT and did not develop a subsequent humoral response whereas the non-AIBD, non-PFIC-2 control had normal BSEP expression and suffered from an unrelated liver condition. Notably, the asymptomatic brother of patient 3 who had the same homozygous PFIC-2 mutation had serum reactivity against BSEP. F-6, monoclonal BSEP antibody. Apparent molecular weight of BSEP is 150 kDa. The full data set of all patients is shown in Supporting Figs. 1 and 2.

BSEP Inhibition by Patient IgG in Vesicular Transport Assays. Transport activity of human recombinant BSEP was measured in *Sf*9 membrane vesicles. For incorporation of antibodies into the vesicular lumen, an adapted freeze-thaw technique[26] was applied. Briefly, purified patient IgG was added to thawed vesicles by gentle pipetting, and the preparation was repeatedly snap-frozen and allowed to thaw slowly on ice. In order to study BSEP transport inhibition from the "intracellular" (extravesicular) side alone, IgG was added as described above and samples were kept on ice as long as those subjected to freeze-thaw cycles.

Rat Liver Perfusion. Animals received care according to the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23, revised 1985; and Australian Psychological Society guidelines). The study protocol was approved by the local authorities. Livers of male Wistar rats (body weight approximately 150 g) were perfused in a nonrecirculating system as previously described.[27] Liver weight was estimated to be 3.5% of total body weight; flow rate was adjusted to 3.7 mL/(g liver*min). TC was perfused throughout the experiment at a concentration of 100 μM in order to saturate bile salt transport. After 30 minutes, Krebs-Henseleit buffer containing 6.57 mg/L of either control or patient IgG was perfused. After 120 minutes, the right ventral liver lobe was harvested and snap-frozen in liquid nitrogen for cryosectioning and immunostaining.

Statistical Analysis. Column diagrams were prepared using Prism (version 5.0a, GraphPad) and significances shown in Fig. 6 were calculated using the Student *t* test function (unpaired; two-sided *P* values).

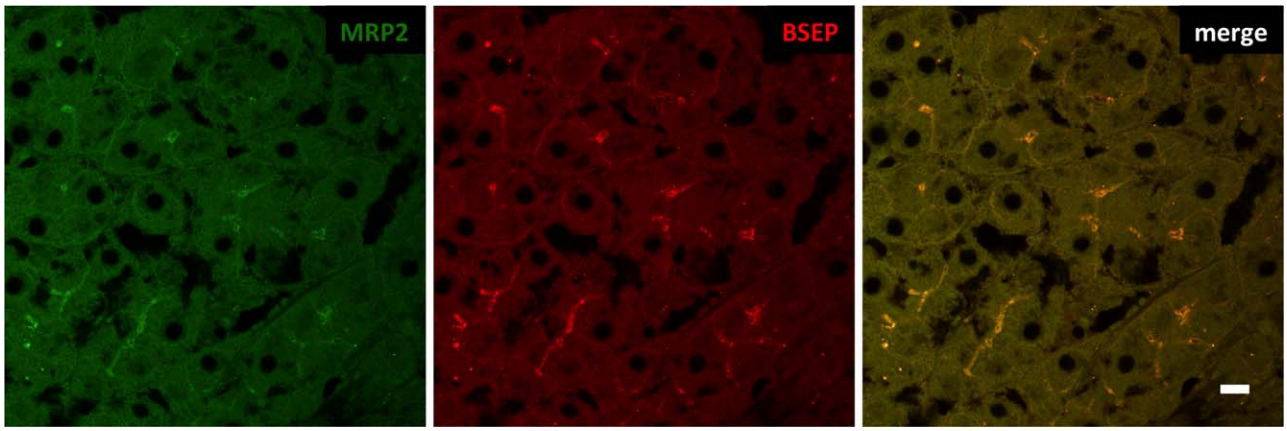

Fig. 2. Canalicular wildtype BSEP expression is clearly detectable in the native liver of AIBD patient 1. The K24 antiserum used for BSEP detection was raised against the carboxy-terminus of BSEP and thus only recognizes the full-length, wildtype gene product from the splice-site-mutated *ABCB11* allele. MRP2 was immunostained as a canalicular marker. Bar = 10 μm.

Results

AIBD Sera Specifically Recognize Human BSEP With a High Titer. We examined sera from 9 patients (1) diagnosed with PFIC-2 or low-GGT intrahepatic cholestasis, (2) that had undergone OLT, and (3) who showed symptoms compatible with disease recurrence. Their clinical and laboratory data are summarized in Table 1. Serum samples taken at the time of symptom recurrence were tested for canalicular immunoreactivity with histopathological sections from normal human liver (Fig. 1A; see Supporting Fig. 1 for the complete data set). All nine post-OLT sera, but not naïve control serum, yielded a canalicular staining pattern, albeit at very different titers (Table 1). Anticanalicular antibodies were detectable at serum dilutions from 1:1,000 up to 1:16,000 for 7 patients (numbered 1 to 7), whereas serum titers of canalicular reactivity were very low with a maximum of 1:100 for 2 patients (numbered 8 and 9). To test for specific reactivity against BSEP, serum samples were then analyzed by immunofluorescence using HEK293 cells transiently transfected with human BSEP-EYFP. All seven high-titer sera recognized recombinant BSEP in transfected cells (Fig. 1B and Supporting Fig. 2), that is, they contained anti-BSEP antibodies. This was further confirmed by immunoblotting analysis, in which only the sera 1-7 specifically detected recombinantly expressed human BSEP (Fig. 1C). Therefore, AIBD was diagnosed in 7 of 9 patients collected by the three criteria stated above (patients 1 to 7).

In contrast, sera from patients 8 and 9 did not recognize recombinant BSEP (immunofluorescence and immunoblotting) and were classified as non-AIBD cases. Patient 8 was a case of PFIC-2 caused by two missense mutations (Table 1) treated by OLT. He suffered from a cholestatic episode with elevated GGT-levels obviously not caused by a humoral anti-BSEP response. Consequently, his serum was used as a PFIC-2, non-AIBD control, which is representative of the majority of tranplanted, asymptomatic PFIC-2 patients.[18] Patient 9 was initially diagnosed as having low-GGT PFIC and was soon after transplanted because of his severe condition. A biopsy of his native liver showed normal canalicular expression of FIC1, BSEP, and multidrug resistance (MDR) 3 (not shown). PFIC-1 and PFIC-2 were eventually excluded by sequencing of the *ATP8B1* and *ABCB11* genes. Hence, his serum was used as a non-PFIC-2, non-AIBD control. Notably, patient 3 had a brother who suffered from the same PFIC-2 mutations as his sister and therefore had to be transplanted at the age of 1.5 years. Although he remained asymptomatic, anti-BSEP antibodies were detected in his serum at a titer of 1:1,600 (western blotting in Fig. 1C) 3 years post-OLT.

AIBD Patients Lack Normal BSEP Expression in Native Liver. Absence of BSEP expression in the native liver has been postulated to be a prerequisite for the development of anti-BSEP antibodies.[18-20,22] In line with this, BSEP was not detectable by immunohistochemistry in native livers of patients 3-7 (Table 1). Patient 2 was homozygous for the nonsense mutation p.Q971X, thus absence of BSEP in his native liver can be assumed. Only in patient 1 was a residual wildtype BSEP expression clearly demonstrated (Fig. 2), as shown by the use of the K24 antibody, which recognized the carboxy (C)-terminus of BSEP. In this patient, one *ABCB11* allele was affected by a c.150+3A>C splice-site mutation, whereas a five-nucleotide insertion

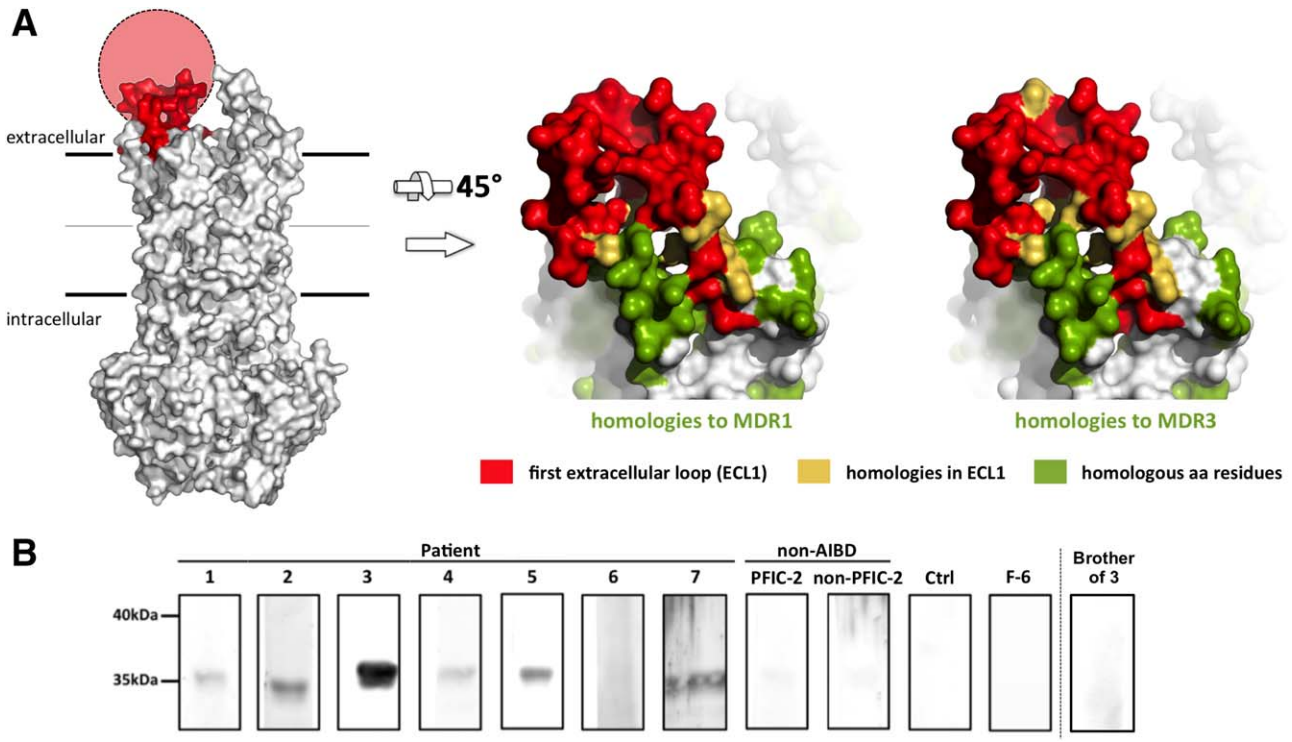

Fig. 3. The first extracellular loop of BSEP is a recurrent, likely critical target of the AIBD response. (A) A homology model of BSEP based on the crystal structure of $SAV1866_2$ suggests that ECL1 (61 amino acid residues [aa]; aa that could be modeled are shown in red; the red circle is intended to give an approximate impression of its actual size) is both a large and potentially accessible epitope. It is unique to BSEP and not found in the closely related MDR3 (ABCB4) or MDR1 (ABCB1). Only a few aa at the edges of ECL1 are conserved between ECL1 and the corresponding loops of MDR1/MDR3 (yellow), whereas a comparison of the remainder of BSEP with its homologs shows higher conservation (green). (B) The ECL1 of BSEP was expressed as a C-terminal EYFP fusion protein in *E. coli* and purified by its N-terminal hexahistidine tag. The epitope is frequently recognized by the immune response of AIBD patients; six of the seven BSEP-reactive antisera detected it when blotted onto nitrocellulose membranes. Interestingly, ECL1 was not detected by the BSEP-reactive serum response from the transplanted, asymptomatic brother of patient 3.

(c.2783_2787dup5) on the other resulted in a frameshift causing the hypothetical protein truncation p.K930Efs79X (Table 1). The remainder of BSEP detected by K24 in his native liver was therefore expressed from the allele carrying the intronic splice-site mutation, encoding the WT protein with its intact C-terminus.

Changes in Immunosuppression, Viral Infections, and Episodes of Acute Rejection May Trigger AIBD. We reviewed the clinical history of all seven AIBD cases in order to identify triggering factors for the development of BSEP-reactive antibodies post-OLT (summarized in Table 1). Cholestasis began to recur from 3 to 108 months post-transplantation. In four of the seven cases, this coincided with a change or decrease of immunosuppressants. In two of these, subsequent transplant rejection was reported. Also, two cases suffered from viral infections (Epstein-Barr virus, hepatitis B virus) at the time of phenotypic disease recurrence. In contrast, neither ischemia reperfusion injury nor biliary injury was documented for any of the AIBD patients or non-AIBD controls, while bouts of acute rejection preceding any potential trigger events were only reported for 1 AIBD patient (see Supporting Table 1).

The First Extracellular Loop of BSEP Is a Common Target of the Antibody Response in AIBD. Because the first AIBD case study[18] identified a sequence within ECL1 as the target of the patient's BSEP-reactive antibody response, we screened all AIBD sera for specific reactivity toward ECL1. Topological considerations predict that ECL1 is the largest extracellular domain of BSEP, with 61 amino acid residues[18,28]; by comparison, ECL2 to ECL6 comprise 4, 20, 20, 4, and 4 residues, respectively. This domain is unique to BSEP and has only minimal homologies to the corresponding loop of MDR1 and MDR3 (MDR1: 18%; MDR3: 21% identity; Fig. 3A). ECL1 was recombinantly expressed in *E. coli* as a fusion protein with a hexahistidine and an EYFP tag (Supporting Fig. 3). Using the purified recombinant epitope, we found ECL1-reactive IgG in nearly all AIBD sera, with the exception of patient 6. In

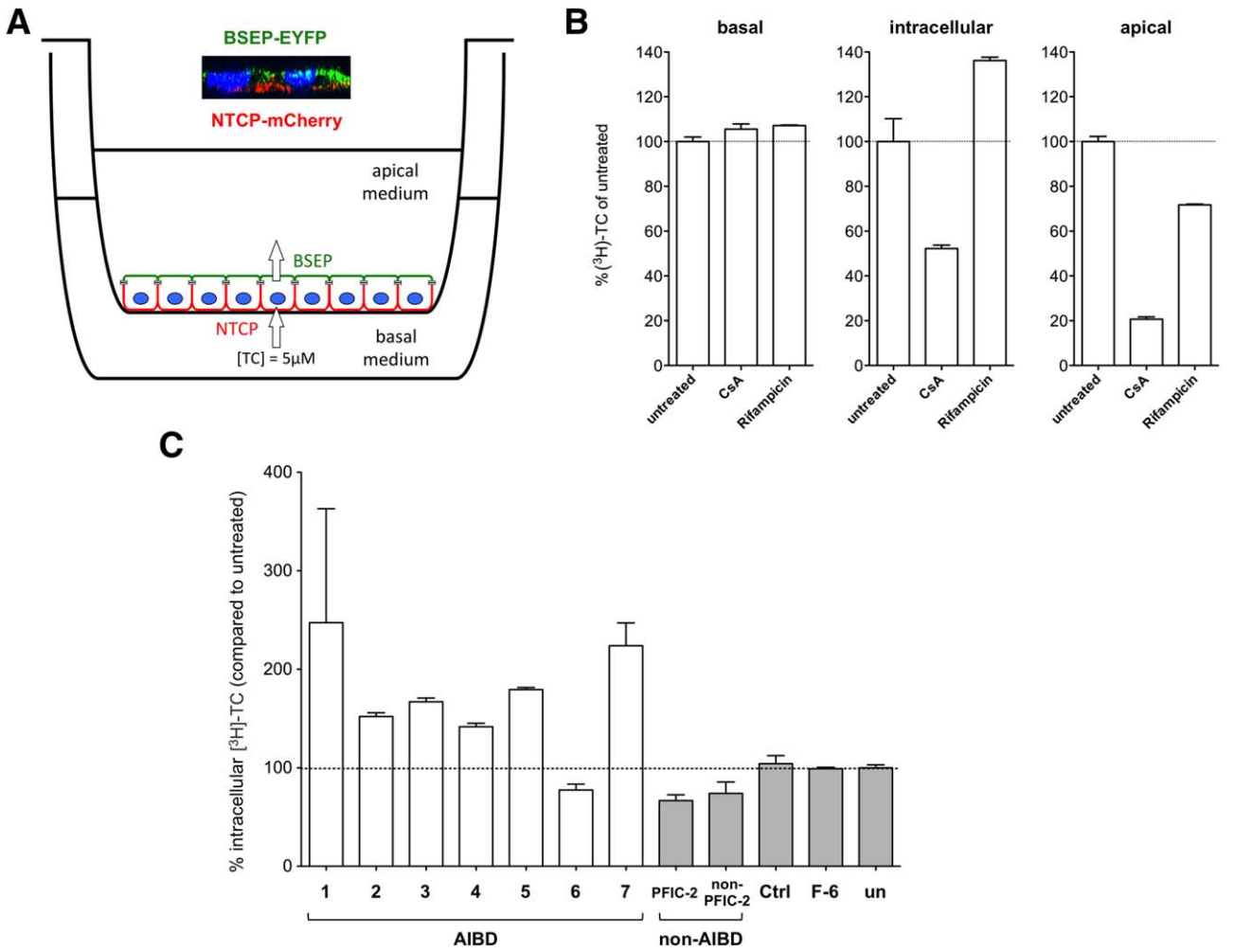

Fig. 4. AIBD sera inhibit BSEP-mediated transport in a polarized cell culture model. (A) Concept of the transepithelial transport assay. Inset, X/Z-section of the NTCP-mCherry (red)/BSEP-EYFP (green) double-transduced LLC-PK$_1$ cell line used in this assay. The intracellular volume of the monolayer is estimated to be around five orders of magnitude less than those of the apical and basolateral compartments. Accordingly, the intracellular equilibrium generated by influx from the source (basal compartment) and efflux to the sink (apical compartment) is much more sensitive to extracellular inhibition of TC efflux by BSEP whereas both the source and the sink compartment do not show significant changes upon apical serum incubation. (B) Inhibition of BSEP and NTCP/BSEP activity by rifampicin and CsA, respectively, indicates functionality of both transporter proteins. Cell monolayers were preincubated for 1 hour with these agents, applied apically. (C) Inhibition of BSEP-mediated transepithelial transport of TC in LLC-PK$_1$ cells by overnight apical incubation with AIBD and non-ABID control sera. Only incubation of cell monolayers with AIBD sera led to an increase in intracellular TC (imported by NTCP from the basal compartment). Notably, serum from patient 6, showing no BSEP inhibition, was the only AIBD patient serum incapable of detecting ECL1 (see Fig. 3B). Columns and error bars represent the mean and standard error of two independent measurements. un, untreated.

contrast, both non-AIBD control sera failed to recognize ECL1 (Fig. 3B). Strikingly, the transplanted (PFIC-2), asymptomatic brother of patient 3 had no ECL1 reactivity in his serum sample whereas his serum antibodies recognized full-length BSEP. The lack of reactivity in all controls also demonstrates that the human IgG pool is devoid of unspecific reactivity against the EYFP domain of the fusion protein.

AIBD Sera Inhibit Human BSEP From the Extracellular Side. The inhibitory capacity of anti-BSEP antibodies was analyzed in a cell- and a vesicle-based transport assay. We generated a double-transduced LLC-PK$_1$ cell line expressing human NTCP at the basolateral membrane and BSEP at the apical membrane (Fig. 4A), resulting in the vectorial transport of [^{3}H]-TC from the basal into the apical compartment (Fig. 4A,B). Untransduced LLC-PK$_1$ cells, as with cells expressing exclusively NTCP or BSEP, showed no significant transepithelial [^{3}H]-TC transport (Supporting Fig. 4). In double-transduced cells, inhibition of both BSEP and NTCP activity by 50 μM of cyclosporin A (CsA) resulted in a reduced intracellular uptake of [^{3}H]-TC, whereas inhibition of BSEP activity alone by 50 μM of rifampicin led to an increase in intracellular [^{3}H]-TC content (Fig. 4B). When the apical face of the double-transduced LLC-PK$_1$ cells was preincubated overnight

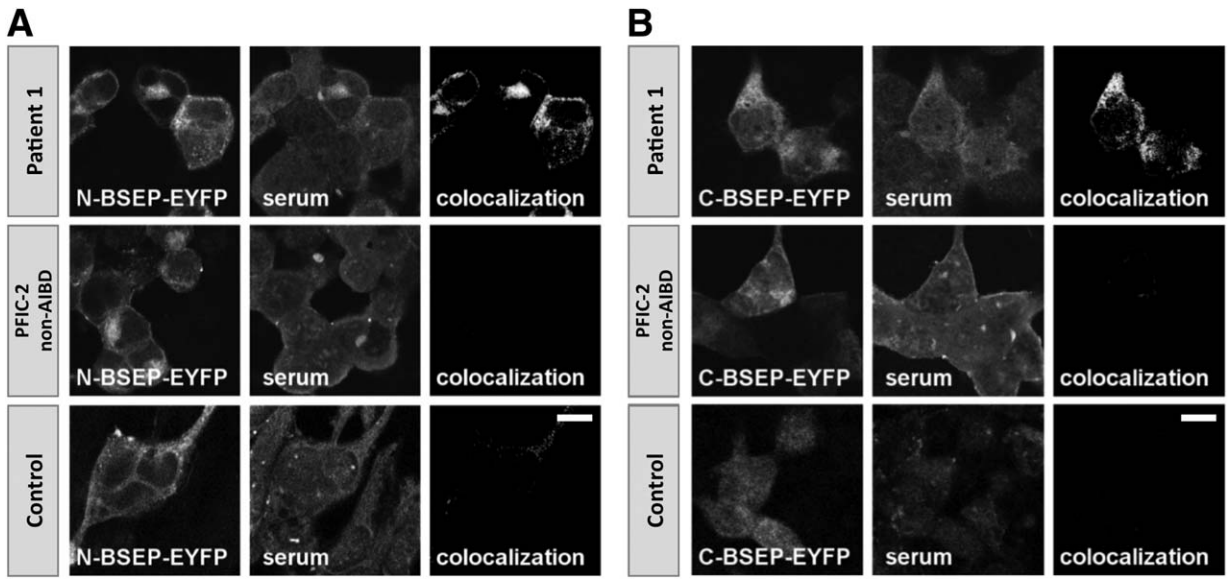

Fig. 5. AIBD sera stain HEK293 cells transiently expressing the N- or C-terminal halves of BSEP. (A and B) As exemplified by patient 1, all AIBD sera except those from patients 2 and 5 recognize both halves; the exceptions show little or no reactivity against the C-terminal half. In contrast, the non-AIBD controls and naïve control serum fail to detect either half of BSEP. Likely owing to its lack of the native N-terminal signal anchor sequence, the C-terminal half is largely retained intracellularly. Bars = 10 μm.

with AIBD sera, intracellular amounts of [^{3}H]-TC increased; this was ascribed to antibody-mediated inhibition of BSEP activity (also see Supporting Fig. 4 for an illustrative comparison of the different transgenic LLC-PK$_1$ cell lines). This BSEP inhibition was observed in the presence of all AIBD sera except patient 6 (Fig. 4C), who lacked serum reactivity against ECL1 (see Fig. 3B). In contrast, BSEP activity was not inhibited by the non-AIBD controls, naïve control serum or the monoclonal anti-BSEP antibody, F-6, which was raised against the N-terminal 180 amino acid residues but did not recognize ECL1.

AIBD Antibodies Detect Multiple Epitopes of BSEP and Inhibit Its Function From Intra- and Extracellular Sides. Whether AIBD antibodies react with one or several distinct domains of BSEP is unknown. To investigate this, we stained HEK293 cells transiently expressing either the N- or C-terminal half of BSEP[18] with patients' sera. As shown in Fig. 5A for patient 1, all AIBD sera recognized the N-terminus of BSEP, which contains ECL1 (for the complete set, see Supporting Figs. 5 and 6). The majority of sera also recognized the C-terminal half of BSEP (Fig. 5B), with two sera (2 and 5) showing little or no reactivity. Non-AIBD sera and the naïve control failed to recognize either the N- or C-terminal BSEP half.

Where sample material sufficed, total IgG from AIBD patient serum was purified for *in vitro* inhibition studies using human recombinant BSEP expressed in insect cell membrane vesicles (Fig. 6). To differentiate between intra- and extracellular inhibition, we measured transport activity in vesicles prepared with or without freeze/thaw cycles (Fig. 6A). By applying repeated freeze/thaw cycles, the antibodies were incorporated into the vesicular lumina, thus exposing both the intra- and extracellular sides of BSEP to patients' IgGs. In vesicles, which were not prepared by freeze/thaw cycles, BSEP was only accessible from the extravesicular side. The variable IgG concentrations reflect variable amounts of available serum, which, in some cases, was limiting (Fig. 6B; see IgG concentrations). Using this assay, we could confirm that all tested AIBD sera demonstrated IgG-mediated BSEP inhibition (Fig. 6B, white bars), ranging from approximately 6.9% to 21.6% per mg/mL total patient IgG. In contrast, the low-concentration IgG from patient 7 only showed a slight inhibition. IgG amounts from patient 6 allowed only for one assay mode, so the freeze/thaw variant was chosen for maximal accessibility of BSEP. In the remaining three AIBD IgG pools (patients 2-4), BSEP inhibition was significantly more effective when the transporter was accessible from both sides (white bars) than when it was accessible from the "intracellular" (extravesicular) side only (gray bars). Taken together, our findings demonstrate that BSEP recognition and inhibition are indeed aspects of a polyclonal antibody response directed against both intra- and extracellular portions of BSEP.

BSEP-Reactive IgG Rapidly Decorates Canalicular BSEP in Perfused Rat Liver. So far, strong IgG deposits in the canalicular space of liver from an AIBD

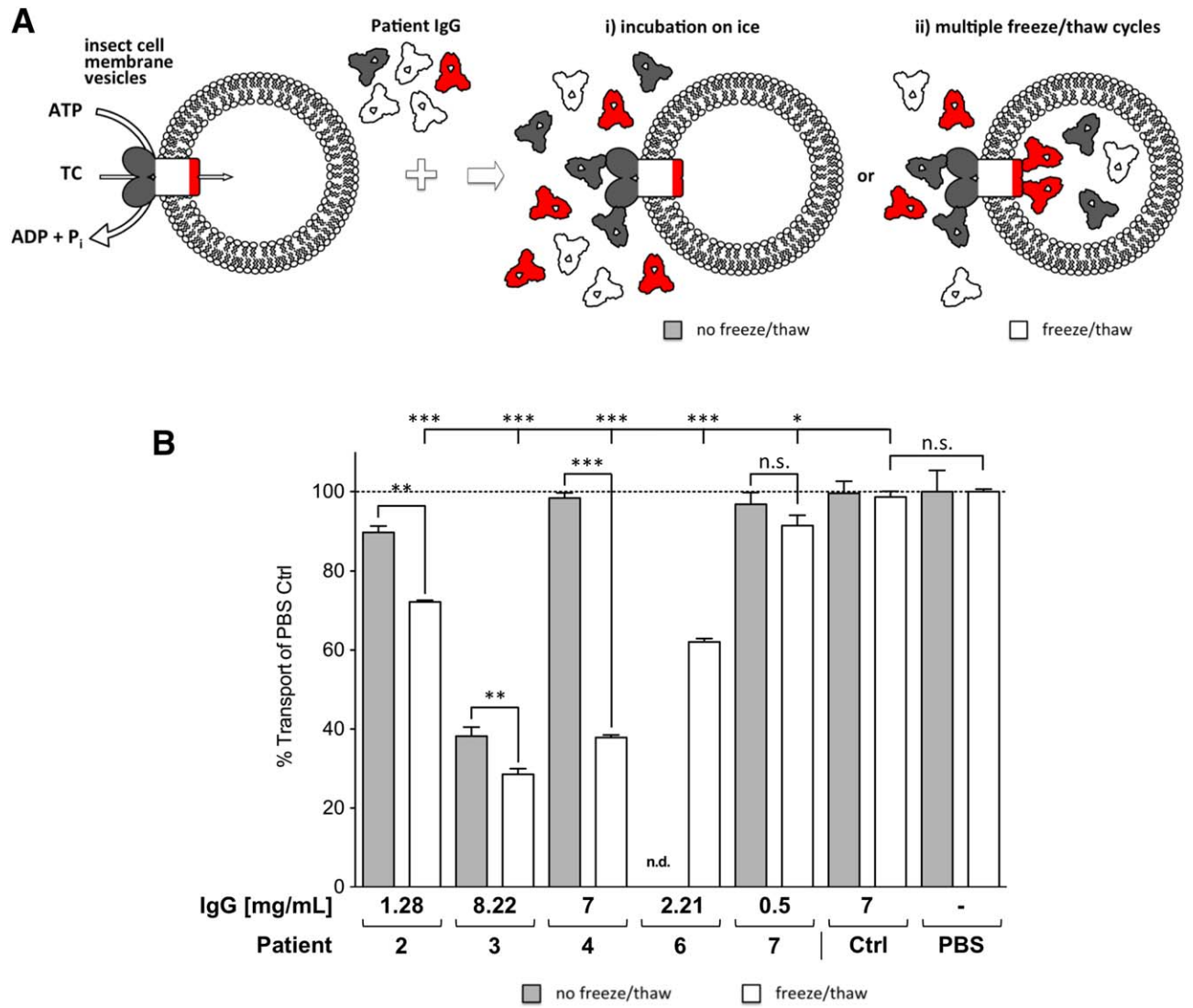

Fig. 6. BSEP-mediated TC transport in a vesicular assay is inhibited from the extra- as well as the intraluminal side. (A) Concept of the different vesicle incubation methods used in this assay. The red and dark gray antibodies recognize extra- and intracellular epitopes of BSEP, respectively. In essence, BSEP may be inhibited either (1) solely from its intracellular side or (2) from both its intracellular and its extracellular side. [^{3}H]-TC uptake by rapid filtration can be measured only using inside-out vesicles such as those shown here, which expose nucleotide-binding domains to ATP-containing buffer. (B) Purified patient IgGs were incorporated into the lumina of *Sf*9 membrane vesicles containing human BSEP by multiple freeze/thaw steps (white columns). Thus, BSEP-reactive antibodies can bind their targets on the outside of assayable (i.e., inside-out) vesicles as well as on the intraluminal ("extracellular") side (see (A, ii). Alternatively, BSEP vesicles were incubated on ice for the same duration with same amounts of patient IgG (gray columns). Here, only extravesicular ("intracellular") parts of BSEP are accessible to patient antibodies (see A, i). Data in (B) represent mean and standard error of three independent measurements and were analyzed using the Student *t* test (unpaired; two-sided *P* values). $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$; n.s., not significant.

patient have only once been described.[18] In our cohort, biopsy materials from allograft livers were available for all AIBD cases except patient 6. We could detect a clear canalicular IgG staining pattern using an anti-human IgG antibody in all available AIBD liver samples (Supporting Fig. 7). In contrast, no IgG was detected in the canaliculi of transplant biopsies from the non-AIBD controls. To find out whether and how fast BSEP-reactive IgG reaches the canalicular space in intact organs, rat livers were perfused *in situ* for 2 hours (Fig. 7). Rat Bsep is highly homologous to human BSEP and it has been shown previously that human AIBD sera recognize rat Bsep.[18,19] Using purified total IgG from patient 4 (16 mg), we observed a distinct, patch-like canalicular staining (Fig. 7B) that was absent when using an equal amount of naïve control IgG (Fig. 7A). This indicates that anti-BSEP IgG antibodies rapidly reach the canalicular space. Of note, we did not observe a

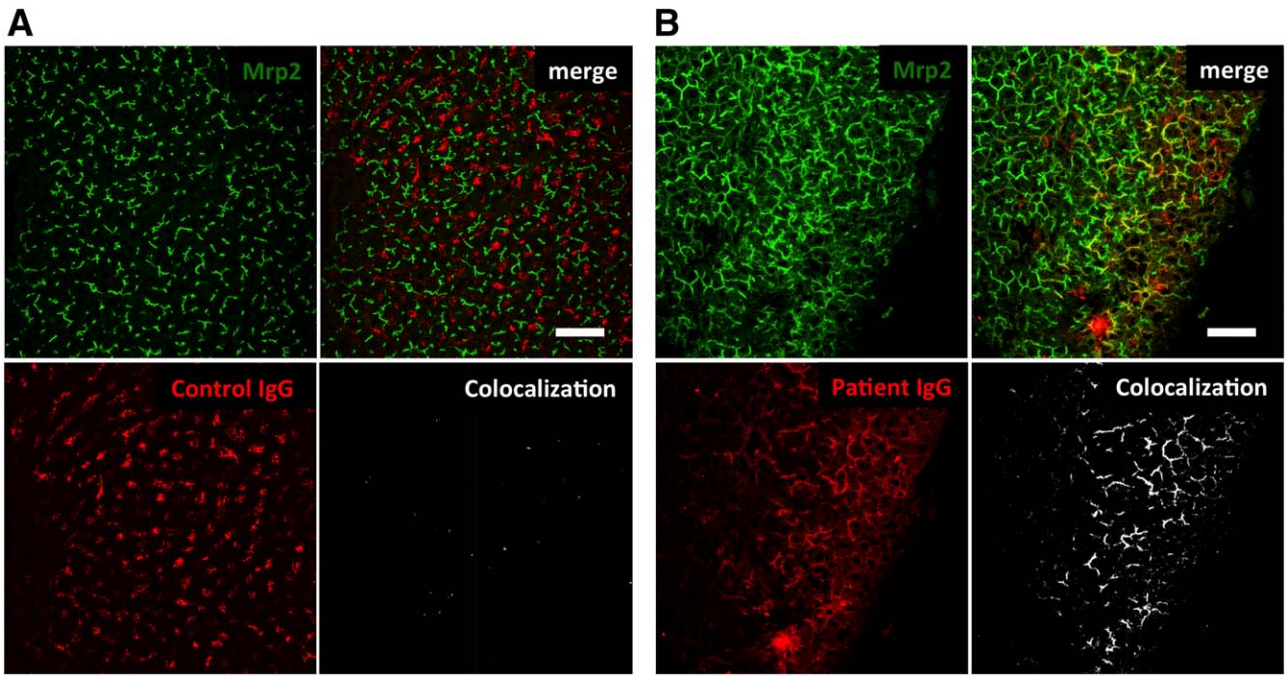

Fig. 7. BSEP-reactive IgG rapidly decorates canalicular BSEP in perfused rat liver. Rat livers were perfused *in situ* for 2 hours with equal amounts of total IgG purified from either (A) a naïve control or (B) AIBD patient 4 (from whom sufficient IgG could be obtained by purification from serum retained on immunoadsorption columns). Only perfusion with BSEP-reactive IgG resulted in a canalicular pattern of colocalization of variable extent (yellow in the merge panels) of human antibodies (red) with the canalicular marker Mrp2 (green). Naïve IgG stained only the sinusoids. Colocalized pixels are shown in white in the lower right panels. Bars = 50 μm.

change in bile salt-dependent bile flow (not shown), which likely is owing to incomplete antibody saturation of canalicular BSEP during the short time of perfusion.

Discussion

The development of anti-BSEP antibodies in PFIC-2 patients post-OLT was first described in 2009 by us and others.[18,19] This study demonstrates, for the first time, the polyclonal nature of the BSEP-reactive antibody repertoire, which targets epitopes on both N- and C-terminal halves of BSEP. Furthermore, anti-BSEP antibodies bind to both intra- and extracellular domains, inhibiting bile salt transport (Fig. 6). However, on intact hepatocytes only extracellular epitopes are accessible to antibodies from the canalicular lumen. Accordingly, only antibodies directed against extracellular structures can inhibit BSEP function *in vivo* and are thus of pathophysiological importance. Notably, Jara et al. demonstrated *in vitro* inhibition of BSEP-mediated transport after incubation of insect cell membrane vesicles with AIBD patient sera.[19] In the rapid filtration assay that they used, the buffer solution bathing the vesicles contained adenosine triphosphate (ATP), magnesium, and the inhibitory antibodies. Only when vesicular membranes are oriented inside-out, exposing the intracellular nucleotide-binding domains of BSEP to ATP, BSEP can transport the radiolabeled substrate into the vesicular lumen (compare Fig. 6A). Hence, the inhibition that Jara et al. demonstrated for three BSEP-reactive sera could only result from binding of IgG reactive toward intracellular BSEP domains. Upon inclusion of BSEP-reactive IgG within the vesicular lumen, we found a significantly higher decrease in transport, indicating that BSEP was inhibited from both the intra- and extracellular sides (Fig. 6B).

Unlike isolated vesicles, the closed polarized LLC-PK_1 cell monolayer only permits binding of antibodies to extracellular epitopes on intact cells. Preincubation of the apical cell surface with AIBD sera led to an increase in intracellular TC concentrations in this transport assay (Fig. 4C). That this effect was moderate may be owing to the small amounts of patient IgGs used, resulting in only partial or incomplete BSEP inhibition. Additionally, the extracellular matrix of the dense LLC-PK_1 cell monolayer may also reduce antibody access to BSEP. However, because the intracellular volume of the monolayer is much smaller than that of both medium-containing compartments, even small changes in TC influx or efflux are expected to result in a measurable change in cellular [^{3}H]-TC content.

A BSEP homology model based on the $SAV1866_2$ ABC transporter structure[29] indicates that ECL1 is the largest domain of BSEP accessible to antibodies from

the extracellular space (Fig. 3A). This domain is exclusively found in BSEP (ABCB11, also called sister of P-glycoprotein), but not in any other of its close relatives such as MDR1 (ABCB1, P-glycoprotein) and MDR3 (ABCB4; Fig. 3A, right panels) and likely has some unique functional role yet to be uncovered. We previously identified this domain as the target of an AIBD patient's anti-BSEP response.[18] Indeed, ECL1 was found to be a recurrent and likely critical target of the AIBD antibody response (Fig. 3). This is also supported by our finding that the serum of patient 6, devoid of reactivity against ECL1, failed to inhibit BSEP from the extracellular side in the transepithelial transport system (Fig. 4). Unfortunately, no biopsy material was available to check this patient's allograft for canalicular IgG deposits. Ultimately, some uncertainty remains whether this patient suffered from "true" AIBD because he eventually died from severe bleeding complications caused by a necrotizing pancreatitis 1.5 years after the detection of anti-BSEP antibodies. The notion of ECL1 as a critical extracellular epitope is further supported by the finding that serum from the asymptomatic, transplanted brother of patient 3 lacked reactivity against this domain while recognizing full-length BSEP (Fig. 3B). Clearly, he was not affected by AIBD. Taken together, which epitopes are recognized determines differences in severity and outcome of AIBD in individual patients.

After activation and proliferation of BSEP-reactive B cells, anti-BSEP antibodies reach the canalicular space of the allograft liver where they exert their inhibitory function. We could previously show that only BSEP-reactive IgG decorates the canalicular membrane.[18] In the study by Jara et al., livers of Wistar rats injected with BSEP-reactive patient serum at 48 and 24 hours before sacrifice showed canalicular staining specific for human IgG that was absent when using normal serum.[19] Strikingly, this canalicular antibody deposition is already detected after 2 hours of perfusion in rat liver (Fig. 7B). Moreover, BSEP-reactive IgG deposits may be considered a general diagnostic feature of AIBD, given that we could find these exclusively in allograft liver biopsy specimens from AIBD patients (Supporting Fig. 7).

Four findings overall strongly suggest a pathophysiological role of BSEP-reactive antibodies in AIBD: (1) recognition of extracellular BSEP epitopes; (2) capacity of antibodies binding to extracellular BSEP epitopes to inhibit BSEP function; (3) targeting of anti-BSEP antibodies to the canalicular membrane *in vivo*; and (4) canalicular antibody deposits in AIBD patients, but not in patients with intrahepatic cholestasis after OLT who lack anti-BSEP antibodies. The relevance of these antibodies as mediators of AIBD is also underlined by the clinical observation that AIBD symptoms are temporarily alleviated by immunoadsorption,[18] which removes antibodies from the circulation, and by treatment with rituximab,[22] a chimeric monoclonal therapeutic anti-CD20 antibody that induces B-cell depletion and is used for the treatment of autoimmune diseases. Rituximab treatment induced remission of cholestasis in patients 1, 4, and 5 in this study. Patient 6 passed away from unrelated complications and thus could not benefit from similar treatment. Successful treatment by rituximab also suggests that affected bile canaliculi must eventually undergo antibody clearance.

It has been speculated that complete absence of BSEP expression is a prerequisite for development of BSEP-reactive antibodies post-OLT.[18,20,30] In line with this, all our patients who developed post-transplant anti-BSEP antibodies carried mutations with predicted severe effects on both *ABCB11* alleles. Specifically, no missense mutations were found among our AIBD patients, but instead only nonsense, frameshift, or splice-site mutations (Table 1). Notably, one AIBD case (patient 1) had demonstrable, albeit strongly reduced, canalicular WT BSEP expression in his native liver (Fig. 1), yet post-OLT developed anti-BSEP antibodies. In general, central tolerance against tissue-specific antigens (TSAs) such as BSEP is obtained by clonal deletion (by apoptosis) of autoreactive T cells in the thymus, whereas autoreactive B cells in bone marrow are controlled both by clonal deletion and receptor editing.[31-33] Whereas the B-cell repertoire is known to contain low-affinity, autoreactive B-cell receptors, which normally broaden the initial humoral immune response, any BSEP-reactive B cell requires a "fitting" T-helper cell for coactivation, which recognizes the same antigen by its T-cell receptor (TCR). In thymus, medullary thymic epithelial cells (mTECs) stochastically express an abundant variety of peptides derived from TSAs. Any TCR binding to these peptide-MHC complexes is removed from the TCR pool along with its T cell by apoptosis. Although small amounts of mature, fully spliced messenger RNA (mRNA) in the presence of the splice-site mutation, c.150+3A>C, could be detected in the native liver of patient 1 (Dröge et al., in preparation), it is well conceivable that mRNA processing within mTECs was ineffective for sufficient peptide production, resulting in impaired clonal deletion of BSEP-reactive T cells. Peripheral tolerance against transplanted BSEP on the other hand would require constant antigen exposure.[34,35] In this context, BSEP may be regarded as a conditionally sequestered antigen. Residing in the canalicular membrane, it remains inaccessible to cellular

components of the immune system and is only released into the circulation upon acute hepatocellular damage, which then may trigger antibody production. Taken together, AIBD should also be considered in transplanted PFIC-2 patients with a residual BSEP expression before OLT upon symptom recurrence.

Some important pathomechanistic questions of AIBD remain to be addressed. It is still unclear how the BSEP-reactive antibodies reach the canalicular space,[30] as is by what mechanism(s) the polyclonal antibody repertoire inhibits BSEP function *in vivo*. Binding to the extracellular side of BSEP may occlude the translocation pore[18] or impose other conformational restrictions that prevent transporter function. Additionally, antibody-mediated cross-linking of BSEP molecules in the canalicular membrane may contribute to functional inhibition by sequestering BSEP into larger aggregates that, in turn, may disrupt the unique structure and asymmetry of this membrane environment. Answers to these questions hold the key to a better understanding of AIBD pathogenesis and to find improved means of treatment or altogether prevent this severe type of acquired cholestasis.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28311/suppinfo.