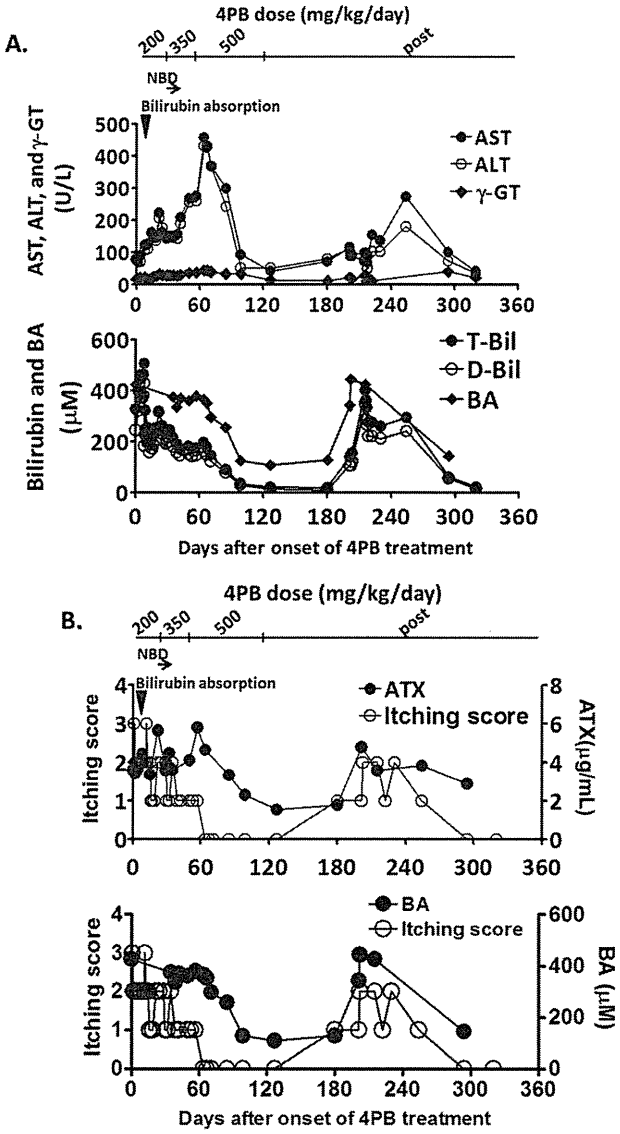

Figure 4 Liver function testing and itching intensity in a BRIC2 patient during and after the course of 4PB therapy. (a) Serum AST, ALT, γ-GT (upper), T-Bil, D-Bil, and BA (lower) levels were monitored during and after the period of 4PB therapy. (b) Correlation of itching scores for the BRIC2 patient with serum concentrations of ATX (upper) and BA (lower) in the patient during and after 4PB therapy. Pruritus severity was scored from 0 (no pruritus) to 4 (cutaneous mutilation, with bleeding and scarring) as described in Methods. The arrow and arrowhead indicate the time point and period when the patient underwent bilirubin absorption and NBD, respectively. γ-GT, γ-glutamyltransferase; 4PB, 4-phenylbutyrate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATX, autotaxin; BA, bile acids; BRIC2, benign recurrent intrahepatic cholestasis type 2; D-Bil, direct bilirubin; NBD, nasobiliary drainage; T-Bil, total bilirubin; WT, wild type.

surface-resident $BSEP^{WT}$ and $BSEP^{E297G}$, the form found most frequently in PFIC2 patients, and interrupted their internalization and degradation, resulting in induction of their expression at the plasma membrane.[16,27,28] Considering that p.D404G and p.V444A, the mutations found in this BRIC2 patient, have an impact on BSEP that is similar to, but milder than, the PFIC2-type mutation p.E297G (Fig. 2),[23,29] it is most likely that 4PB therapy increased the cell surface expression of BSEP in this BRIC2 patient through the same mechanism as in PFIC2 patients with the p.E297G mutation. This is consistent with the quantitative polymerase chain reaction analysis showing that transcriptional processing of $BSEP^{D404G}$ was unaffected by the treatment with 4PB (Fig. 3a).

NBD has been employed successfully for resolving cholestatic episodes in six of seven BRIC patients.[8] Immediate, complete and long-lasting symptomatic relief was elicited. In contrast, no apparent therapeutic effect of 1 week of NBD was observed for the BRIC2 patient in this study. NBD eliminates bile constituents including bile salts, bilirubin and pruritogens, and interrupts their enterohepatic circulation, thereby improving liver function and intractable pruritus. Therefore, it may be less effective in BRIC patients such as one in this study in whom biliary bile salt excretion and bile flow formation were severely disrupted. Medical therapy with 4PB that restores decreased hepatocanalicular expression of BSEP, biliary secretion of bile salts and bile flow formation is a reasonable and promising avenue for treatment of the cholestatic attacks in patients with this type of BRIC.

Treatment with 4PB may also have therapeutic potency for episodic intrahepatic cholestasis in specific BRIC1 patients, because an *in vitro* analysis has shown that treatment with 4PB partially restored the decreased expression of ATP8B1 caused by *ATP8B1* mutations naturally occurring in these patients.[30] Therefore, it is conceivable that 4PB therapy could relieve the cholestatic episodes in patients with BRIC1 who retain protein activity of ATP8B1 per se. Furthermore, even in the BRIC1 patients whose liver functions are barely improved by treatment with 4PB, intractable itching, the main complaint in the cholestatic attacks of patients with BRIC1, should disappear, because the antipruritic potency of 4PB on cholestatic itching was indicated in our previous study.[24] In PFIC1 patients, regardless of the lack of improvement in liver functions assessed by biochemical and histological analysis, therapy with 4PB significantly relieved sustained refractory pruritus, made it possible for the patients to sleep well, and thereby helped improve their quality of life and that of their families.[24] The relief of intractable itching by 4PB

therapy in the patient in this study might have been achieved by not only the improvement in liver function but also by its antipruritic effect, because the itching resolved prior to the improvement in liver function tests and to the decrease in the factors suspected to be causally associated with cholestatic pruritus. At present, the mechanism underlying the relief of cholestatic pruritus by 4PB therapy remains to be elucidated. Future studies will provide evidence regarding the clinical utility and safety of treatment with 4PB in BRIC1 patients and a better understanding of the mechanisms responsible for the effects of 4PB therapy on cholestatic pruritus.

Our study indicates that 4PB is the first mechanism-based drug against intrahepatic cholestasis in BRIC2 patients. Therapy with 4PB markedly improved liver function and relieved intractable itching during a cholestatic episode in a patient with BRIC2. Together with the fact that all the BRIC2-type mutations previously analyzed show a similar impact on BSEP to that of the p.D404G and p.V444A, mutations in this patient, this suggests that 4PB therapy should be effective in the majority of BRIC2 patients. Future clinical studies should be undertaken to validate the favorable outcome and safety of 4PB treatment in more patients than was possible in this study. If its usefulness is confirmed, 4PB therapy could become the preferred choice, instead of the existing medical and invasive therapy, for attenuating cholestatic episodes in BRIC2 patients. Clinical trials will be required to determine the utility and safety of 4PB as a therapy for BRIC1 and other diseases with cholestatic pruritus.

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V 肝癌の病理・病態

肝 芽 腫

Hepatoblastoma

谷川 健　　鹿毛政義

Key words：肝芽腫，病因，病期，診断，予後

1 定　義

肝芽腫は小児期にみられる肝腫瘍であり，胎生期の肝細胞に類似した上皮性細胞の増殖からなる．種々な分化度を示し，ほかに類骨組織，紡錘形細胞などの間葉成分が種々な割合で混在する[1,2]．現在，我が国における組織分類はWHO組織分類[1]を基盤としている．

2 疫　学

肝芽腫はまれな腫瘍であり，100万-150万人に1人の発生で，肝芽腫の80-90％は5歳未満に発生するとされている[1]．日本小児外科学会悪性腫瘍委員会による集計[3]では，小児肝悪性腫瘍の登録数は過去20年間(1993-2012年)に734症例である．そのうち肝芽腫は584症例(79.6％)であり，肝芽腫は小児肝悪性腫瘍の大部分を占めている．年間27-46症例(平均37症例/年)であり，著しい増加傾向はないと思われる．男女比は1-1.5：1でやや男児に多い傾向にある．年齢分布は2歳以下に多い傾向にある．

3 病　因

1）遺伝子異常

肝芽腫の発癌機構はいまだ解明されていない．成人肝癌のようにウイルス感染との関連は少ない．Beckwith-Wiedemann症候群(BWS)や家族性大腸ポリポーシス(FAP)で，肝芽腫発生が多く認められる．肝芽腫の発生に関与していると考えられている遺伝子異常を示す．

a. β-catenin遺伝子，APC遺伝子

FAP家系に肝芽腫が発生することがいわれている．FAP患者にAPC遺伝子異常がみられることから，Wntシグナル伝達経路が発癌に関与している可能性が考えられるようになった．β-cateninやAPCはWntシグナル伝達経路の構成分子である．Wntシグナル伝達経路は，細胞質や核に存在するβ-cateninのタンパク質量を調節し，細胞の増殖，分化を制御している．APCやβ-cateninの遺伝子変異によって，Axin/APC/β-catenin/GSK-3βの複合体形成あるいはβ-cateninのリン酸化などが行われずに，β-cateninが細胞質や核に蓄積し，癌化すると考えられている[4]．また，β-cateninがリン酸化を受ける部位はexon 3である．肝芽腫でこの部位に点突然変異や欠失が報告されている[5]．これによって，β-cateninの分解が阻害され，発癌に関与しているのではないかと推察されている．

b. Insulin-like growth factor II(IGF2)遺伝子

BWSでは11p15の異常が認められる．11p15

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にはH19/IGF2遺伝子が含まれている．この異常により，IGF2遺伝子のloss of imprintingが起こり，IGF2の過剰発現が生じる．IGF2は成長ホルモン依存性に乏しく，インスリン様作用が強い因子であり，細胞増殖に関連している．IGF2遺伝子過剰発現が腫瘍増殖に関わっていると推察されている[6]．

2）低出生体重児

肝芽腫と低出生体重児との関連が知られている[7]．低出生体重児の生存例が増加するにつれ，肝芽腫の発生例も増加している．これらの患児における肝芽腫の発生には，周産期治療に関連する何らかの要素が関わっているのではないかといわれている．低出生体重児に発生する肝芽腫は，病期が進行しやすく予後が不良である傾向がある．

4 症　　状

肝芽腫に特異的な症状はなく，腹部腫瘤触知，腹部膨満，腹痛，嘔吐などの症状で発見される．腫瘍が大きくないときには無症状であり，診断時には巨大な腫瘍となっていることもある．遠隔転移は肺が主であるが，まれに骨，脳などに転移を認めることもある．また，腫瘍の腹腔内破裂により出血性ショックに陥ることもある．

5 診　　断

1）血液・生化学検査

貧血，白血球増多や血小板増多を認めることがある．AST/ALTは正常範囲か軽度上昇している．高コレステロール血症がみられることもある．LDHは軽度上昇ないし高値を示す．

腫瘍マーカーとして，αフェトプロテイン(AFP)がほとんどの肝芽腫症例で陽性となり，多くは異常高値を示す．診断で有用であるとともに，経過観察や治療効果判定にも有用である．診断において，新生児，乳児は血清AFP値が生理的に高く，月齢での正常値より高値であるかどうかを判定する必要がある．AFPは癌胎児性タンパクの一つであり，胎生期の卵黄嚢や肝臓で生理的に産生される．肝芽腫だけでなく，肝細胞癌，卵黄嚢癌でも高値を示す．AFPには分画があり，AFP-L1，AFP-L2，AFP-L3の3つがある．肝芽腫ではAFP-L3が上昇する．卵黄嚢癌ではAFP-L2が上昇し，鑑別可能である[7]．

2）画像診断[8]

画像検査の意義は，①ほかの肝腫瘍との鑑別，②占拠区域の同定(完全切除の可否判定)，③遠隔転移の有無を調べることである．

a. 胸腹部単純X線検査

腹部単純X線写真で肝腫大がみられる．病巣に一致して石灰化像が認められることがある．特異的所見に乏しく，それのみでは診断はできない．

肝芽腫は初診時に肺転移をみることもあるので，胸部X線で転移の有無を調べる必要がある．

b. 超音波検査

簡便で非侵襲的である．線維性被膜を有する例は辺縁が明瞭となる．超音波像では通常，不均一なパターンを示す．上皮成分の多い腫瘍は均一な低エコー像であることが多い．石灰化に対応した高エコー像や壊死に対応した無エコー像がみられることがある．さらに，肝静脈/下大静脈，門脈などの脈管との関係を調べる．

c. Computed tomography(CT)

単純CTで，腫瘍は周囲肝に比べ低吸収域を呈する．石灰化や隔壁形成による分葉構造がみられることが多い．造影CTの所見は一定していない．

肝芽腫は肺転移を伴うことが多いので，術前，術後に転移の有無を調べる必要がある．

d. Magnetic resonance imaging(MRI)

MRIで，腫瘍はT1強調画像で低信号，T2強調画像で高信号となる．分葉構造を示すこともある．出血巣，壊死巣，石灰化巣を有する場合は内部不均一となる．

e. 血管造影

血管造影で，腫瘍はhypervascularであり，血管の新生，進展，およびencasementを認める．

3）肉眼診断[1,2]

肝芽腫は肉眼的に，塊状型，多結節型，びまん型に分類される．塊状型が最も多いとされて

いる．腫瘍の割面は，分葉構造を有し，黄褐色ないし灰赤褐色を示すことが多い．線維性隔壁，壊死や出血，胆汁色を呈する部など多彩な所見がみられる．周囲肝とは境界明瞭で，偽被膜を有することが多い．背景肝に肝硬変はみられない．

4）組織診断[1,2]

a. 胎児型

腫瘍は2-3層からなる細胞索を形成し，増殖している．類洞が認められることが多い．腫瘍細胞は胎児期の肝細胞に類似した立方形，多面形の均一な細胞であり，正常肝細胞に比べて小型である．細胞質は豊富で淡明あるいは好酸性を呈している．核は円形で小さく，形態は均一である．

b. 胎芽型

腫瘍は管状やリボン状あるいはロゼット様配列を示し，増殖している．細胞間の結合性が低い部分も多い．腫瘍細胞は多形，短紡錘形で，細胞質は少なくN/C比が高い．腫瘍細胞は一般に正常肝細胞に比べて小さいが，大小不同がみられる．核は濃染し，分裂像もまれではない．類洞は部分的に認められる．vascular lakeや造血巣がしばしば認められる．

c. 大索状構造

腫瘍細胞が10層以上に多層化した索状配列，あるいは集塊状構造をとり，増殖している．島状に分けられた組織は類洞によって囲まれ，肝細胞癌に似た所見を示す．腫瘍細胞は大型で，大小不同を示し，大型の核を有する．核小体が明瞭なことが多い．核分裂像もしばしば認められる．

d. 未分化小細胞型

腫瘍は不規則に増殖し，特別な配列はみられない．腫瘍細胞は小型で，特徴に乏しい円形ないし卵円形であり，未熟な細胞である．腫瘍細胞は細胞質に乏しく，核はクロマチンに濃染し，核が目立つ．

e. 上皮・間葉混合型

上皮性成分とともに間葉成分が混合した腫瘍である．間葉成分には線維性組織，類骨様組織，紡錘形組織などがある．類骨様組織は好酸性基質の中に多形性ないしは紡錘形の細胞がみられ，類骨に似た組織像を示す．紡錘形細胞は正常な間質細胞とは異なる長・短の紡錘形細胞で異型性や多形性を示す細胞群である．上皮性腫瘍細胞巣に接して存在することが多く，上皮性細胞からの移行も認められる．

f. 類奇形腫混合型

肝芽腫組織内に奇形腫様組織が含まれるものである．その組織には，神経芽腫に類似した未分化神経組織やメラニン色素細胞などの神経上皮性細胞あるいは神経内分泌細胞，さらに粘液細胞や線毛を有する細胞を含む腺組織，角化を伴う扁平上皮などがある．

5）組織鑑別診断

a. 大索状型肝芽腫と肝細胞癌

特に年長児肝芽腫症例は肝細胞癌との鑑別が困難であることが多い．正常肝細胞に比して，肝芽腫細胞は小型～やや大型で，肝細胞癌細胞は大型である．肝芽腫は肝細胞癌に比べ，細胞質が乏しく，核異型も軽度である．肝芽腫の大部分は5歳以下，肝細胞癌の大部分は10歳以上で発生する．基礎疾患などの臨床所見を含めて，総合的に判断する．

b. 胎芽型肝芽腫と神経芽細胞腫未分化型

両者とも細胞質に乏しく，ほとんど裸核状である．神経芽細胞腫未分化型では腫瘍細胞間に神経細線維はみられず，びまん性増殖を示し，鑑別は困難である．免疫染色で，肝芽腫ではHP-1，cytokeratin 8，18などが陽性，神経芽細胞腫未分化型ではneuron specific enolase(NSE)，neurofilament(NF)，neural cell adhesion molecule(NCAM)などが陽性となり，鑑別点となる．

6 病期(図1)

肝芽腫の予後は病期と関連があり，完全切除可否の判定にも病期が重要である．我が国では現在，SIOPEL(Childhood Liver Tumors Strategy Group)で使用しているPRETEXT(Pre-Treatment Extent of Disease)を用いている[9]．治療開始前に病期を決めるもので，腫瘍占拠区域により肝切除の難易度を示した分類である．

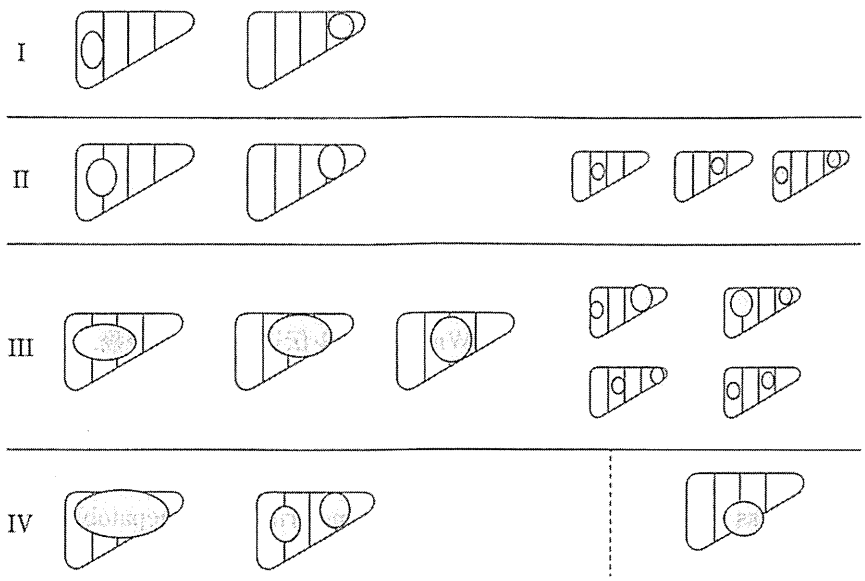

図1 肝芽腫の病期分類

[JPLT-2の分類(SIOPEL PRETEXT[9]を参考)]

病期I: 腫瘍は完全に切除され肝に限局している．肝被膜あるいは偽被膜に腫瘍が浸潤していても外側表面には達しておらず完全切除されている．肝切除縁の主血管は巻き込まれていない(腫瘍内血管が巻き込まれていてもよい)．転移は認めない．

病期II: 腫瘍は肉眼的に切除されているが，組織学的な腫瘍残存(positive margin)があること，あるいは腫瘍破裂または手術時の腫瘍のspillageがあるもの．転移は認めない．

病期III: 切除断端を越え肉眼的に腫瘍が残存する不完全切除，あるいは腹部のリンパ節が巻き込まれている場合，あるいは遠隔転移はないが被膜が破裂した場合．

病期IV: 血行性転移(肺，肝，骨，脳など)あるいは肝浸潤に関連のない腹腔外あるいは骨盤腔外のリンパ節へ転移がある場合．

占拠区域のほか，尾状葉腫瘍，肝外腹腔内腫瘍，腫瘍個数，腫瘍破裂，リンパ節転移，門脈浸潤，下大静脈・肝静脈浸潤などの項目もある．

7 予　後

1) 病期と予後

肝芽腫の予後は完全切除の可否が大きく影響するといわれている[10]．腫瘍の状態は前述のPRETEXTで評価される．近年では，日本小児肝癌スタディグループ(JPLT)により，系統だてられた化学療法のプロトコールが確立され，不完全切除症例においても治療成績は大きく改善している．

2) 組織所見と予後

組織型と予後については，胎児型肝芽腫，特に純胎児型肝芽腫は予後良好であるといわれている[11]．また，大索状型肝芽腫は予後不良とされている[12]が，一定の見解は得られていない[10]．上皮・間葉混合型の予後は悪くないとされている[10]．しかし，腫瘍組織の大半を占める上皮成分が予後に大きく影響すると考えられ，上皮・間葉混合型では上皮成分の優勢度を付記することが推奨されている[13]．

細胞分裂像と予後に有意な関連があると報告されている[10,13]．また，脈管浸潤と予後に有意な関連はないとされている[10]が，血行性転移が時にみられることがあり，今後の検討が望まれる[14]．

3) 臨床検査所見と予後

予後不良因子として，AFP値が100 ng/mL以下[15]，病期IV，腹腔内肝外病変，腹腔内出血，遠隔転移，リンパ節転移，門脈浸潤，肝静脈/下大静脈浸潤など[9]が挙げられている．

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Natural Killer Cells Regulate T Cell Immune Responses in Primary Biliary Cirrhosis

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The hallmark of primary biliary cirrhosis (PBC) is the presence of autoreactive T- and B-cell responses that target biliary epithelial cells (BECs). Biliary cell cytotoxicity is dependent upon initiation of innate immune responses followed by chronic adaptive, as well as bystander, mechanisms. Critical to these mechanisms are interactions between natural killer (NK) cells and BECs. We have taken advantage of the ability to isolate relatively pure viable preparations of liver-derived NK cells, BECs, and endothelial cells, and studied interactions between NK cells and BECs and focused on the mechanisms that activate autoreactive T cells, their dependence on interferon (IFN)-γ, and expression of BEC major histocompatibility complex (MHC) class I and II molecules. Here we show that at a high NK/BEC ratio, NK cells are cytotoxic for autologous BECs, but are not dependent on autoantigen, yet still activate autoreactive $CD4^+$ T cells in the presence of antigen presenting cells. In contrast, at a low NK/BEC ratio, BECs are not lysed, but IFN-γ production is induced, which facilitates expression of MHC class I and II molecules on BEC and protects them from lysis upon subsequent exposure to autoreactive NK cells. Furthermore, IFN-γ secreted from NK cells after exposure to autologous BECs is essential for this protective function and enables autoreactive $CD4^+$ T cells to become cytopathic. *Conclusions*: NK cell-mediated innate immune responses are likely critical at the initial stage of PBC, but also facilitate and maintain the chronic cytopathic effect of autoantigen-specific T cells, essential for progression of disease. (HEPATOLOGY 2015;62:1817-1827)

There have been considerable efforts to define the effector mechanisms that lead to biliary cell destruction in primary biliary cirrhosis (PBC).[1-4] Indeed, such work has defined the epitopes recognized by autoimmune $CD4^+$ and $CD8^+$ T cells and autoantibody and has led to the thesis that a multilineage response against E2 component of pyruvate dehydrogenase complex (PDC-E2), the immunodominant autoantigen in PBC, is an essential component of disease pathogenesis.[5-11] Our laboratory has suggested, both from work in human PBC and animal models of autoimmune cholangitis, that innate immune responses shape acquired immune responses in the pathogenesis of cholangitis.[12-16] Indeed, there is increasing recognition

Abbreviations: Abs, antibodies; APCs, antigen presenting cells; BECs, biliary epithelial cells; DCs, dendritic cells; DMEM, Dulbecco's modified Eagle's medium; ECs, endothelial cells; ELISA, enzyme-linked immunosorbent assay; E/T, effector/target ratio; FCS, fetal calf serum; hepatitis C virus; HLA, human leukocyte antigen; HPLC, high-pressure liquid chromatography; IFN, interferon; IL, interleukin; LMC, liver-derived mononuclear cell; mAb, monoclonal antibody; LT-β, lymphotoxin beta; MHC, major histocompatibility complex; NK cells, natural killer cells; PBC, primary biliary cirrhosis; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PDC-E2, E2 component of pyruvate dehydrogenase complex; SD, standard deviation; SpMCs, spleen mononuclear cells; Th, T helper; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha.

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that an adaptive immune response requires significant contributions from innate immunity, both in a normal immune response and in a breach of tolerance.[13,17-21]

PBC is an organ-specific autoimmune disease without evidence of global defect in either innate or adaptive responses.[22,23] It is particularly noteworthy that the liver, which normally functions to restore and maintain tolerance, itself becomes the victim of an autoimmune response, leading directly to portal inflammation and destruction of biliary epithelial cells (BECs).[24-26] Moreover, PBC reoccurs after liver transplantation, and recent data suggest that, although BECs express Toll-like receptors (TLRs) and can function as antigen presenting cells (APCs), the BEC is itself only a victim of an immune attack based upon its own unique apoptotic properties, namely, that the major mitochondrial autoantigens of PBC remain immunologically intact within an apoptotic bleb.[24,27-30] Our laboratory has demonstrated the ability to isolate BECs and phenotypically defined individually isolated liver-infiltrating mononuclear cells.[13,14,31] We have taken advantage of this technology and the availability of human liver to study in detail the role of activated natural killer (NK) cells in the induction of autologous biliary epithelium cytotoxicity. We propose, based on our data, that the critical bridge that links innate immune response with the adaptive effector autoimmune responses characteristic of PBC is activation of NK cells. Indeed, we demonstrate herein that appropriate pretreatment of NK cells can modulate BEC destruction. Furthermore, NK cells directly influence the ability of autoreactive $CD4^+$ T cells to destroy PDC-E2 peptide antigen-pulsed BECs. Finally, interferon (IFN)-γ appears to play a central role in this switch from innate to acquired immunity, highlighting the important function of T helper (Th)1 cytokines in PBC. We submit that further studies on the switch mechanisms between innate and adaptive immunity have the potential to alter the effector mechanisms that lead to continued portal inflammation.

Materials and Methods

Subjects and Protocol. Explanted liver and spleen from a total of 8 patients were used throughout this study, and, in all cases, their liver-derived mononuclear cell (LMC) populations and intrahepatic BECs were isolated and defined as described below. These eight explanted organs included three3 livers with PBC and five livers with hepatitis C virus (HCV) infection. We should emphasize, as described below, that there were no significant differences in the results derived from the liver mononuclear cell or the BEC population from either PBC or hepatitis C, and, ultimately, all data were combined. Furthermore, the observation that liver samples from PBC and hepatitis C can be used for the current study is consistent with our thesis that the BEC is a victim as well as the established data that PBC reoccurs post-transplantation. All eight livers were derived from patients with end-stage cirrhosis, but in whom there was no evidence of an acute liver process or any unrelated disease. The 3 patients with PBC were known to have high titer antimitochondrial antibodies, and the diagnosis was based on established criteria.[32] In all cases, livers were obtained after informed consent and all protocols were approved by the research ethics committee of Kyushu University (Fukuoka, Japan).

Isolation of Intrahepatic BECs and Endothelial Cells From Liver. LMC populations were isolated as previously described.[7,12] Briefly, liver specimens were first digested with 1 mg/mL of collagenase type I. Cells from digested tissue were purified to obtain LMCs as previously described.[8] Adherent cells within LMCs were isolated after incubation of cells overnight in tissue-culture plates. The adherent cell population was maintained in tissue culture until cells reached full confluence, usually by day 14, and the nonadherent cell population aspirated, washed, and cryopreserved in medium containing 7.5% dimethyl sulfoxide and stored in liquid nitrogen. BECs were separated from adherent cells using CD326-conjugated (epithelial cell adhesion molecule) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) specific for epithelial cells. Cells were then resuspended in medium consisting of a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum (FCS), epithelial growth factor (10 ng/mL), cholera toxin (10 ng/mL), hydrocortisone (0.4 μg/mL), tri-

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iodothyronine (1.3 μg/L), transferrin (5 μg/mL), insulin (5 μg/mL), adenine (24.3 μg/mL), and 10 ng/mL of hepatocyte growth factor (R&D systems, Minneapolis, MN) and cultured.[12,31] Purity of cells was verified by immunohistochemistry using antibodies (Abs) against cytokeratins 7 and 19 (Dako, Glostrup, Denmark), and only cultures that were >90% positive for these cytokeratins and >95% viable (as determined by trypan blue) utilized for the studies are reported herein. Endothelial cells (ECs) were separated from BECs and adherent cells using CD31 microbeads specific for ECs. Cultures used in the studies herein were between four and six passages to exclude the potential loss of phenotype after prolonged *in vitro* culture. The methods used herein have all been previously described.[13,14,16,31]

Cytotoxicity of NK Cells Against Autologous BECs and ECs. All assays were performed with autologous cell populations; the ability of NK cells to lyse BECs or ECs was assessed using a previously described 8-hour ^{51}Cr release assay against autologous BECs or ECs.[12,32] Briefly, detached BECs or ECs were labeled with 2 μCi/well of ^{51}Cr (Amersham Biosciences, Amersham, UK) overnight, washed 3× in medium, and 5×10^3 cells dispensed into individual wells of a 96-well round-bottomed plate. To prepare effector NK cells, spleen was mechanically disrupted and dissociated cells were filtered through a 150-μm mesh and separated by Ficoll centrifugation to obtain spleen mononuclear cells (SpMCs).[33] As previously described,[7,14] SpMCs used for the assay were stimulated for 3 days with the TLR3 ligand, poly (I:C), and TLR4 ligand, lipopolysaccharide, each at an optimal concentration of 10 μg/mL. Activated spleen NK cells were purified using an NK cell isolation kit (Miltenyi Biotec). Purity of the isolated NK cell population was >90%, as determined by flow cytometry with anti-CD56 monoclonal Ab (mAb; Miltenyi Biotec) and viability >95%. Isolated activated NK cells were added to triplicate wells with BEC or EC target cells at an effector/target cell ratio of 50:1, 10:1, 2:1, and 0.5:1 in a total volume of 200 μL in complete RPMI medium. Controls consisted of triplicate wells containing target cells cultured alone and target cells incubated with 10% Triton X-100 to determine spontaneous and maximal ^{51}Cr release, respectively. After incubation of cocultures of the effector with target cells for 8 hours, 100 μL of supernatant fluid was collected from each well and counted and the percentage of specific ^{51}Cr release calculated as (cpm of experimental release − cpm of spontaneous release)/(cpm of maximal release − cpm of spontaneous release) × 100 (%). In a modified cytotoxicity assay, BECs were incubated with or without autologous NK cells at an NK/BEC ratio of 0.5 for 24 hours in the presence or absence of either IFN-γ (final concentration: 0.4, 2.0, or 10 ng/mL) or mAb to NKG2D (final concentration: 25 μg/mL; BioLegend, San Diego, CA), IFN-γ, or human leukocyte antigen (HLA) class I (final concentration: 50 μg/mL; R&D systems). Cytotoxicity was quantitated as described above.

Analysis of Cellular Debris Released From the Cytotoxicity Assay. To analyze the contents of the cellular debris after NK cell-mediated lysis of BECs or ECs, we first seeded BECs or ECs at a concentration of 1×10^5 cells/well in six-well plates in complete BEC medium, a 1:1 mixture of Ham's F12 and DMEM, supplemented with 5% FCS, epithelial growth factor (10 ng/mL), Cholera toxin (10 ng/mL), hydrocortisone (0.4 μg/mL), tri-iodo-thyronine (1.3 μg/L), transferrin (5 μg/mL), insulin (5 μg/mL), adenine (24.3 μg/mL; all from Sigma-Aldrich, St. Louis, MO) and hepatocyte growth factor (10 ng/mL; R&D Systems), or endothelial-specific medium (HuMedia-EG2) that included cell growth factors (Kurabo, Osaka, Japan). Activated NK cells were added to each well at 5×10^6 cells/well (effector/target ratio [E:T] ratio = 50) for BECs and ECs, and 1×10^6 cells/well (E:T ratio = 10) for BECs, then cultured for 24 hours. Subcellular fragments of BECs or ECs were isolated by filtration and ultracentrifugation. Briefly, cell-culture supernatant fluid was collected and two additional centrifugation steps (500*g* for 5 minutes) were performed to remove remaining cells and cell fragments. Supernatant fluid was then passed through a 1.2μm nonpyrogenic, hydrophilic syringe filter. BECs fluid from an E:T ratio of 50 were also used unfiltered as a positive control. After centrifugation at 100,000*g* for 30 minutes, the pellet containing microparticles was resuspended in lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysis was performed for 30 minutes on ice. Protein content of the samples was determined by bicinconic acid assay using a Nanodrop ND-1000 ultraviolet-visible Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Each sample (20μg) was diluted in loading buffer and subjected to standard sodium dodecyl sulfate polyvinylidene fluoride membrane separation. PDC-E2 was detected using our standardized mAb, clone 2H-4C8, as previously described.[34]

Proliferation Assays of T-Cell Clones That Respond to PDC-E2 163-176. We took advantage of our previously well described and well-characterized T-cell clones that respond to PDC-E2 163-176. First, the PDC-E2 163-176 peptide, GDLLAEIETDKATI, was synthesized by F-moc chemistry using a peptide synthesizer (Model Synergy; Applied Biosystems Inc., Foster

City, CA). The peptide was purified by reverse-phase high-pressure liquid chromatography (HPLC) to a degree of >90% as determined by HPLC. Cryopreserved T-cell clone TCC independent 1 that is specific for PDC-E2 163-176 was thawed, tested for viability, and our standardized proliferation assays performed.[35]

For antigen presentation, we used monocyte-derived dendritic cells (DCs) prepared from HLA-DR53-positive healthy donors, as described previously, with minor modifications.[36] Briefly, peripheral blood mononuclear cells (PBMCs) isolated using Ficoll-Hypaque were allowed to adhere overnight. After removing the nonadherent cell fraction by gentle washing with phosphate-buffered saline (PBS), the remaining adherent cells were maintained in complete RPMI 1640 medium with 1,000 U/mL of human recombinant granulocyte macrophage colony-stimulating factor and 500 U/mL of human recombinant interleukin (IL)−4 (R&D Systems) for 7 days. At the end of the 7-day incubation period, nonadherent cells were recovered as DCs after gentle washing. DCs as antigen presenting cells (APCs) were seeded at 2×10^6 cells/well in 24-well round-bottomed plates and incubated in the presence or absence of the microparticles from BECs or ECs (250 μg/mL at final concentration) for 24 hours. For positive control, DCs were pulsed with PDC-E2 163-176 peptide (10 μg/mL at final concentration). Thence, DCs were harvested, washed, and irradiated (3,000 rad). The CD4 T-cell clone was seeded at 5×10^4 cells/well in 96-well round-bottomed plates with irradiated HLA-DR53-matched DCs (5×10^4 cells/well) that were pre-pulsed or not pulsed with the microparticles or peptide for 72 hours in the presence of 1 μCi/well of ^{3}H-thymidine during the final 12 hours. Cells were then harvested and ^{3}H-thymidine incorporation measured in a beta scintillation counter.

Cytokine Analysis. For analysis of IFN-γ secreted by the T-cell clone, T cells were seeded at 5×10^5 cells/well in 48-well plates with irradiated HLA-DR53-matched DC (5×10^5 cells/well) as APC with PDC-E2 163-176 peptide (10 μg/mL at final concentration) or the microparticles from BEC or EC (250 μg/mL at final concentration) for 24 hours, and the supernatants harvested. For the analysis of cytokines secreted from NK cells, activated NK cells were cocultured with BECs in six-well plates with serial numbers of NK cells and BECs in complete BEC medium for 24 hours, and the supernatants harvested. Concentrations of cytokines in harvested supernatants were evaluated by a sandwich enzyme-linked immunosorbent assay (ELISA), using a combination of unlabeled and biotin- or enzyme-coupled mAbs to IFN-γ, tumor necrosis factor alpha (TNF-α), or lymphotoxin beta (LT-β; R&D Systems).

Cytotoxic Assay of Autoreactive T Cells. Cytotoxic activity of PDC-E2-specific CD4 T cells was assessed using the T-cell clone, TCC independent 1, as effector cells and HLA DR53-matched BECs as target cells. BECs were preincubated with activated autologous NK cells at an NK/BEC ratio of 0.5 for 24 hours. NK cells were removed by washing 3× with PBS. The adherent BEC population was harvested and dispensed into a 96-well round-bottomed plate at 5×10^3 cells/well. BECs were incubated with either PDC-E2 163-176 peptide or a control peptide at 10 μg/mL overnight. T-cell clones were then added at T/BEC ratios of 50:1, 10:1, 2:1, and 0.5:1, and the cytotoxicity assay described above was performed.

Flow Cytometry. To analyze surface expression of HLA molecules, BECs were stained by indirect immunofluorescence using optimal concentrations of mAb to HLA-ABC, E, and DR (BioLegend) before or after incubation with NK cells at an NK/BEC ratio of 0.5, in the presence or absence of IFN-γ at serial final concentrations, for 24 hours. Stained cells were analyzed by flow cytometry.

Statistical Analysis. All experiments were performed in triplicate, and results are presented as mean ± standard deviation (SD). Comparisons between groups were performed by Student *t* test. All analyses were two-tailed, and *P* values <0.05 were considered significant. Statistical analyses were performed using Intercooled Stata software (version 8.0; Stata Corp LP, College Station, TX).

Results

Activated NK Cells Induced Release of PDC-E2 From Autologous BECs. We first examined the cytotoxicity of activated NK cells on autologous BECs or ECs. NK cells isolated from TLR3-L- and TLR4-L-stimulated SpMCs produced significant cytotoxicity when cocultured with autologous BECs or ECs (Fig. 1A); this lysis was regulated by NKG2D and its receptors (Fig. 1B). Importantly, intact PDC-E2 was detected in the unfiltered or filtered lysates obtained from BECs, but not in the filtered lysates of ECs (Fig. 1C). Of note, filtration removed large cell debris resulting in isolation of microparticles. These results indicate that undegraded PDC-E2 is presented in microparticles released from NK-lysed BECs, but not in the lysed ECs. A PDC-E2-specific CD4 T-cell clone underwent proliferation in response to these BEC-derived microparticles (Fig. 1D), and furthermore, IFN-γ production of these T cells

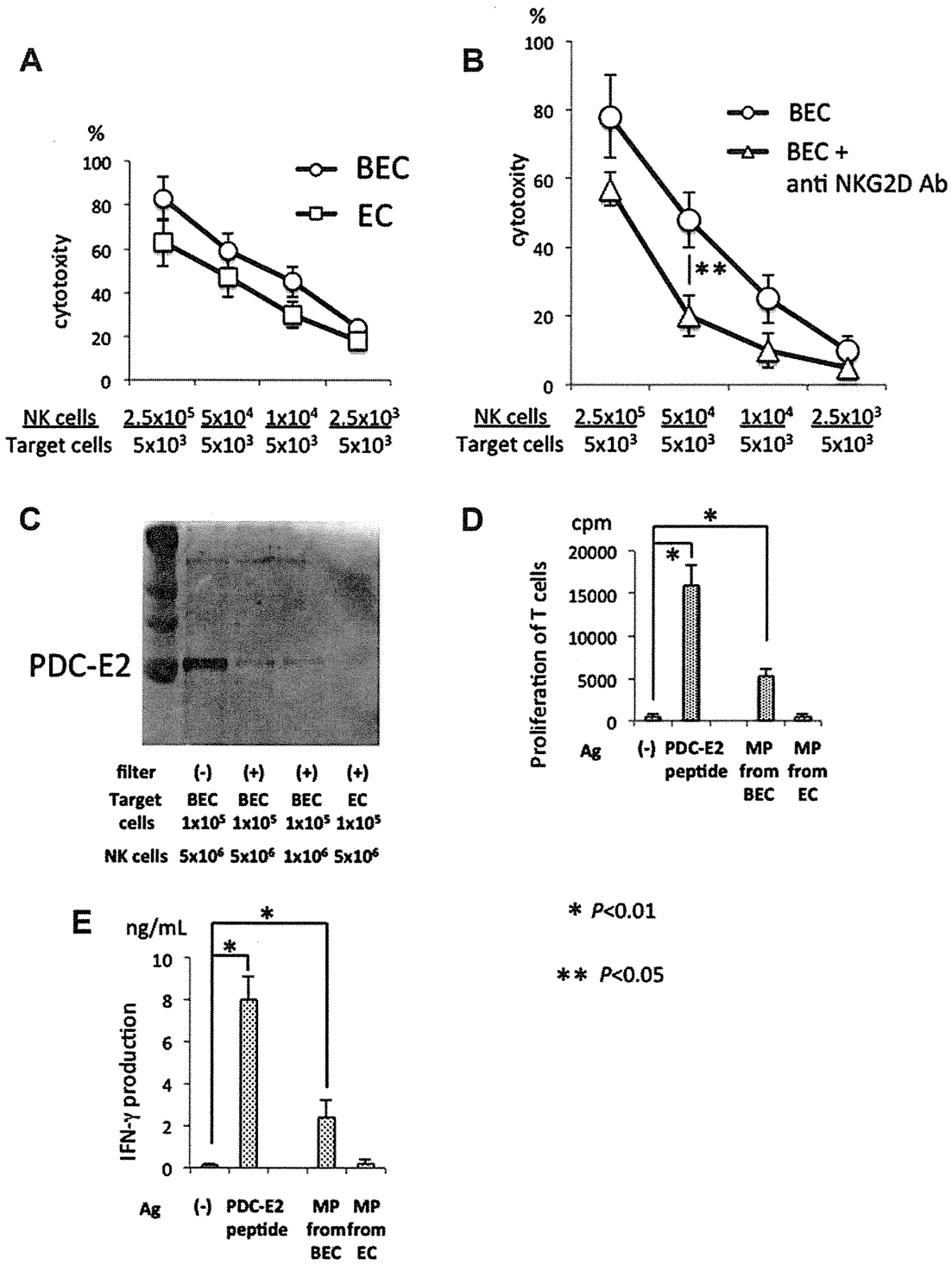

Fig. 1. Activated NK cells cause damage of autologous BECs and release of autoantigen-containing microparticles. (A) Cytotoxicity of activated NK cells against autologous BECs and ECs. SpMC isolated from 2 PBC patients and 5 non-PBC patients were cultured *in vitro* with a mixture of TLR3-L and TLR4-L for 3 days, washed, and then used for isolation of NK cells, which was assayed for cytotoxicity against autologous BEC or EC target cells at serial E/T ratios in a standard ^{51}Cr release assay. The assay was performed in triplicate and results expressed as mean ± SD. Representative data from 1 PBC patient are shown. (B) Activated NK cells were added to cocultures, in triplicate, to bring the NK/BEC ratio to the indicated levels for a standard ^{51}Cr release assay, in the presence or absence of NKG2D Ab. (C) Western blotting analysis for detecting PDC-E2 using a mAb against PDC-E2. The whole lysate of NK-lysed BECs, or microparticles (MP) isolated by filtration and ultracentrifugation from cell lysates recovered after induction of cytolysis with E/T ratio of 50 or 10, were analyzed. (D) Isolated microparticles (MP) were resuspended in PBS and sterilized by filtration, then used as stimulating antigen in a standard ^{3}H-TdR proliferation assay for the PDC-E2-specific $CD4^{+}$ T-cell clone, TCC independent 1.[31] For the purpose of control, the T-cell clone was incubated with the PDC-E2 163-176 peptide (P) or without the peptide (-). HLA DR53-matched irradiated monocyte-derived DCs were included in the assay as APCs. (E) Production of IFN-γ by the PDC-E2-specific $CD4^{+}$ T-cell clone stimulated with microparticles from BECs or ECs as described in (C). Results are presented as mean ± S.D. *Significant differences ($P < 0.01$) between Ag (-) versus PDC-E2 peptide or MP from BECs; **significant differences ($P < 0.05$) between anti-NKG2D Ab treatment is present or absent.

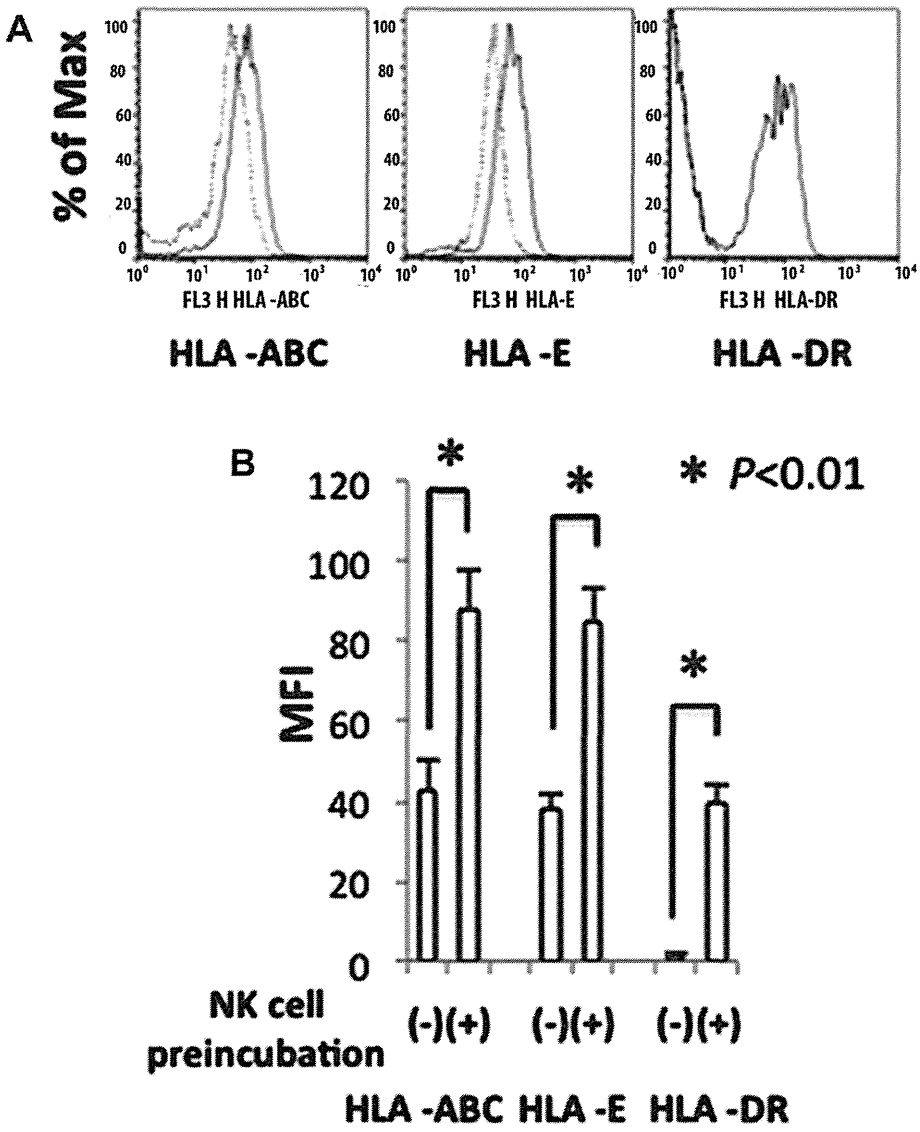

Fig. 2. Effect of NK cells on expression of MHC molecules on BECs. BECs were cocultured with or without activated NK cells for 24 hours at an NK/BEC ratio of 0.5. Levels of cell-surface HLA-ABC, -E, and HLA-DR were assessed by flow cytometry. (A) Representative fluorescence-activated cell sorting plots. (B) Mean fluorescence intensity (MFI) of staining for MHC molecules on BECs with (+) or without (-) preincubation with NK cells.

correlated with their proliferation (Fig. 1E). Hence, although both BECs and ECs were destroyed by NK cells, the microparticles from the lysed BECs differ from those from ECs in terms of their content of the autoantigen PDC-E2 and their ability to activate PDC-E2-specific CD4 T cells.

Phenotypic Changes of BECs Induced by NK Cells. Next, we examined the influence of NK cells on the cocultured BECs. For BECs not incubated with NK cells, we detected expression of HLA-A, B, C, and E, but not HLA-DR (Fig. 2A). After incubation of BECs with NK cells at an NK/BEC ratio of 0.5, the majority of BECs were not lysed and the levels of these major histocompatibility complex (MHC) class I molecules and HLA-DR increased significantly (Fig. 2A,B), indicating that NK cells induced or enhanced expression of the HLA class I and II molecules on cocultured BECs at low NK/BEC ratio.

Cytokine Expression of NK Cells Induced by BECs. We next evaluated the production of cytokines by activated NK cells cocultured with BECs. In agreement with a previous report,[37] activated NK cells produce TNF-α and IFN-γ at different NK/BEC ratios (Fig. 3A), but did not produce LT-β (data not shown). The level of secreted IFN-γ increased with the number of BECs (Fig. 3B). When activated NK cells were not cocultured with BECs, the amount of secreted IFN-γ was negligible (Fig. 3B). These results indicate that BECs induced a production of IFN-γ from NK cells.

NK Cell-Derived IFN-γ Modulates HLA-Dependent NK Cytotoxicity to BECs. NK cells lysed autologous BEC efficiently at higher NK/BEC ratios (Fig. 1A). Reduction of this ratio from 50 to 0.5 in the coculture cytotoxicity assay resulted in diminished BEC lysis. If BECs were pretreated with small numbers of NK cells at an NK/BEC ratio of 0.5, subsequent

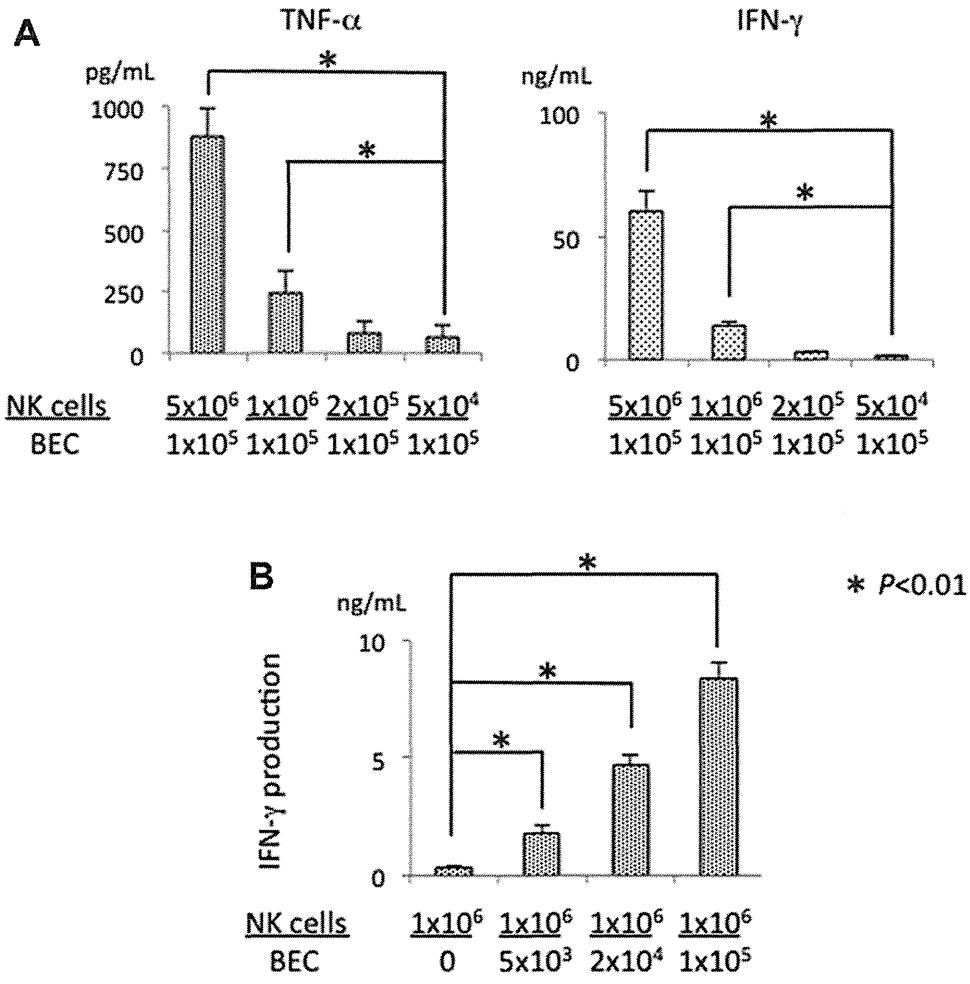

Fig. 3. BEC-induced TNF-α and IFN-γ production from activated NK cells. (A) Activated NK cells were cocultured with autologous BECs (1×10^5 per well) in triplicate at serial NK/BEC ratios for 24 hours. Results are presented as mean ± SD. *Significant differences ($P < 0.01$) between NK cells: 5×10^4 versus 1×10^6 or 5×10^6. (B) Activated NK cells (1×10^6 per well) were coincubated with BECs for 24 hours. Concentrations of TNF-α and IFN-γ in the conditioned medium were measured by ELISA. Results are presented as mean ± SD. *Significant differences ($P < 0.01$) between BECs: 0 versus 5×10^3, 2×10^4, or 1×10^5.

addition of NK cells to higher E/T ratios did not result in lysis of BECs under the same assay conditions (Fig. 4A). To examine whether the protective effect of NK pretreatment is mediated by cytokines produced by NK cells in the presence of autologous BECs, IFN-γ or TNF-α were added to BECs that were not pretreated with NK cells, followed by the NK/BEC coculture cytotoxicity assay. Addition of TNF-α at a concentration of 10 ng/mL did not affect NK cell cytotoxicity against autologous BECs (data not shown). In contrast, addition of IFN-γ significantly suppressed lysis of BECs, which was reduced with an IFN-γ concentration of 0.4 ng/mL and further reduced increased amounts of IFN-γ (Fig. 4A,B). Addition of IFN-γ also resulted in induction of HLA-DR and increased levels of MHC class I molecules on the surface of BECs (Fig. 4C). Addition of anti-IFN-γ mAb to BECs pretreated with NK cells abolished the protection effect of NK pretreatment (Fig. 4A). In addition, blocking HLA class I by adding class I–specific mAb to NK-pretreated BECs also abolished the protective effect of NK pretreatment (Fig. 4A). Taken together, these results suggest that exposure of NK cells to autologous BECs induced IFN-γ production from NK cells, which, in turn, enhanced the expression of class I MHC molecules on BECs and protected the latter from cytotoxicity of NK cells.

Cytotoxicity of Autoreactive CD4$^+$ T-Cell Clone Against BEC Is Enhanced by NK Cells. Our previous work demonstrated that PDC-E2 peptide-specific CD4$^+$ T-cell clones were cytotoxic against HLA-matched BECs pulsed with PDC-E2 peptide in the presence of IFN-γ.[38] Herein, we note that in the absence of exogenous IFN-γ, PDC-E2-specific CD4$^+$ T cells were unable to lyse PDC-E2 peptide-pulsed BECs, even at high T/BEC ratios (Fig. 5). However, when PDC-E2 peptide-pulsed BECs were preincubated with NK cells at an NK/BEC ratio of 0.5, T-cell clones were capable of lysing the target BEC at T/BEC ratios ranging from 50 to 2 (Fig. 5). The effect of NK pretreatment was abolished by the addition of mAb to IFN-γ at all these T/BEC ratios (Fig. 5). These data indicate that NK cells were capable of enhancing the cytotoxicity of autoantigen-specific CD4$^+$ T cells against autologous BECs.

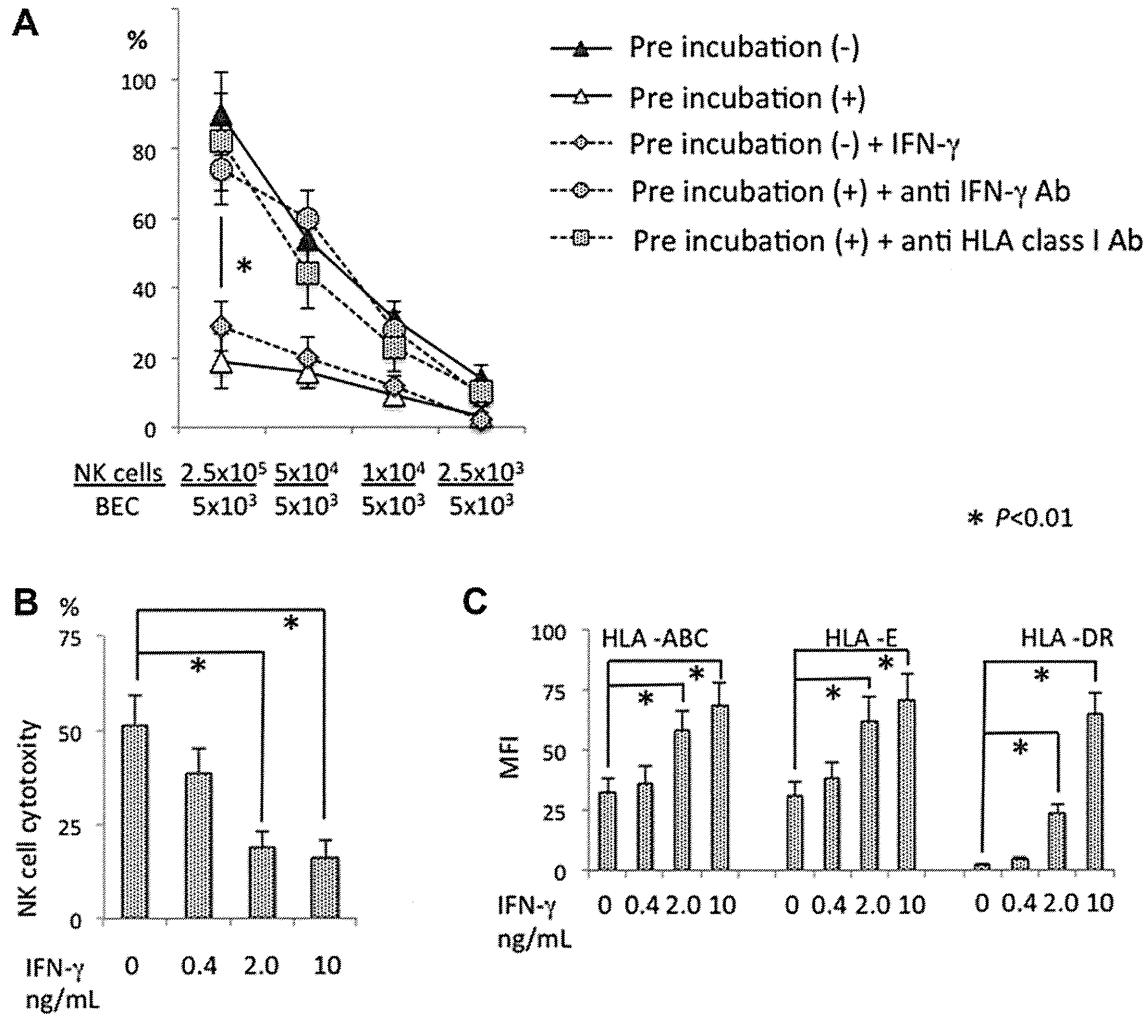

Fig. 4. IFN-γ and HLA class I modulated NK cell cytotoxicity against BECs. BECs were mixed with activated autologous NK cells at an NK/BEC ratio of 0.5 and incubated for 12 hours during preincubation (+). After preincubation, activated NK cells were added to cocultures, in triplicate, to bring the NK/BEC ratio to the indicated levels for a standard ^{51}Cr release assay, in the presence or absence of anti-IFN-γ Ab or anti-HLA class I Ab. BECs without NK cell preincubation (-) were also used as target cells with or without addition of IFN-γ. Results are presented as mean ± SD. *Significant difference ($P < 0.01$) between preincubation (+) versus preincubation (-), preincubation (-) versus preincubation (-) + IFN-γ, preincubation (+) versus preincubation (+) + anti-IFN-γ Ab, or preincubation (+) versus preincubation (+) + anti-HLA Ab. (B) BEC not preincubated with NK cells were cocultured with autologous activated NK cells at an NK/BEC ratio of 50 for a standard ^{51}Cr release assay in the presence of IFN-γ at the indicated concentrations. Results are presented as mean ± SD. *Significant differences ($P < 0.01$) between IFN-γ 0 versus 2.0 or 10. (C) BECs not preincubated with NK cells were cocultured for 24 hours in the presence of IFN-γ at the indicated concentrations, then analyzed for expression of MHC class I and II molecules by flow cytometry. Results are presented as mean ± SD. *Significant differences ($P < 0.01$) between IFN-γ 0 versus 2.0 or 10.

Discussion

In organ-specific autoimmune diseases, target cells modify immunological responses and contribute to pathology. BECs in PBC express cell-surface markers, including HLA class II,[39] CD40,[40] and DR5,[41] in addition to chemokine (C-X3-C motif) ligand 1,[42] that serve as an adhesion molecule; in general, these molecules enhance immunological response. In contrast, BECs in PBC also produce high levels of PG-E2 that suppress immunological responses.[12] In our previous work, we reported that the cytotoxic activity of hepatic NK cells from patients with PBC was increased compared to NK cells from liver-diseased controls against autologous BECs.[31] In the work herein, there were no statistically significant cytotoxic differences between PBC and HCV patients, but the sample size was relatively small and hence we expanded our investigation of the interactions between NK cells and BECs in more detail. We isolated NK cells from SpMCs after activation by TLR ligands and IL-2 stimulation; the number of activated NK cells in the groups herein were similar (data not shown). More important, we demonstrate that at high NK/BEC ratios, NK cells attack BECs, resulting

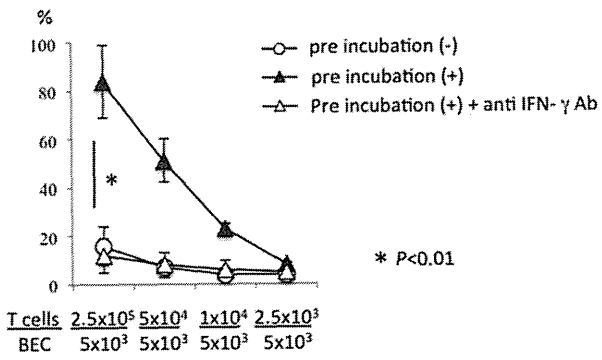

Fig. 5. NK cell pretreatment up-regulated cytotoxicity of autoreactive $CD4^+$ T cells against BECs. BECs, pulsed with the PDC-E2 163-176 peptide, were mixed with (+) or without (-) activated autologous NK cells at an NK/BEC ratio of 0.5 and incubated for 24 hours. The PDC-E2-specific $CD4^+$ T-cell clone, TCC independent 1, was then added as an effector cell at the indicated E/T ratios to BECs in triplicate, followed by a standard ^{51}Cr release assay in the presence or absence of anti-IFN-γ Ab. Results are presented as mean ± SD. *Significant differences ($P < 0.01$) between preincubation (+) versus preincubation (-) or preincubation (+) + anti-IFN-γ Ab.

in release of autoantigens from injured BECs, which, in turn, activate autoreactive T cells in the presence of APCs (Fig. 1); at low NK/BEC ratios, NK cells do not directly damage BECs, whereas IFN-γ secreted from NK cells induce expression of HLA class II on BECs (Fig. 4C), which leads to destruction of BECs by autoreactive $CD4^+$ T cells (Fig. 5). In contrast, we confirmed the destruction of ECs by autoreactive $CD4^+$ T cells after exposure at low NK/autologous EC ratios (data not shown). We speculate that TLR ligands, NK cells, and, of course, autoreactive $CD4^+$ T cells surround BECs, but not ECs, within the microenvironment of a PBC liver. Importantly, we demonstrate that at low NK/BEC ratios, IFN-γ produced from NK cells inhibits direct cytotoxicity of NK cells toward autologous BEC (Fig. 4A,B). Of particular interest, destruction of BECs and ECs by NK cells is neither PBC specific nor PDC-E2 specific (Fig. 1A), suggesting that autoreactive T-cell-mediated acquired immunity becomes the dominant immunological process only in established PBC that follows the initial nonspecific innate immune attack that leads to breakdown of self-tolerance. This is supported by the finding that in established PBC, not only Th17 cells, but also Th1 cells infiltrate into the lesions of chronic nonsuppurative destructive cholangitis with high levels of IFN-γ production derived from Th1 cells.[43]

In addition to IFN-γ that regulates expression of HLA class I and II and plays a role in the interaction between innate immunity and acquired immunity as reflected herein, it is well known that IFN-α, primarily produced by plasmacytoid DCs, leads to enhanced self-reactivity in several autoimmune diseases.[44] Previously, we demonstrated that IFN-α is necessary for activation of NK cells in their destruction of BECs.[31] As with IFN-γ, IFN-α is also known to influence HLA expression.[45,46] However, preincubation of BECs with IFN-α did not affect damage of BECs mediated by NK cells (data not shown). Owing to the fact that IFN-α promotes activation and maturation of conventional DCs, it is likely that type I IFN will enhance the antigen-specific autoreactive T-cell response. The role of type 1 IFNs in pathogenic acquired immunity in PBC should be studied in the future.

The hallmark of acquired immunity in PBC are the T- and B-cell responses targeted to PDC-E2. Because glutathiolation does not occur in apoptotic BECs, PDC-E2 is not degraded by caspases in PBC.[29] As shown in the current work, PDC-E2 in apoptotic BECs is presented in an immunologically intact form in microparticles, which can be internalized by APCs by the process of endocytosis[27,30] and presented to antigen-specific T and B cells, resulting in activation of these acquired immune cells after the initial attack of BECs by autologous NK cells in the presence of professional APCs. It is currently not known whether other parenchymal cells, such as hepatocytes, can also be damaged by NK cells. This should also be examined in future studies, and, indeed, it is possible that subsequent to the action of NK cells on autologous ECs, autoantigens released from apoptotic ECs will be immediately digested and therefore not presented by APCs. In such a scenario, autoreactive responses would be reduced. Finally, we note that a weakness of the study herein is the small numbers of PBC livers and the surrogate use of HCV livers. However, our thesis, as discussed above, is that BECs are victims of an effector response, a hypothesis supported by our previous data and by the recurrence of PBC after liver transplants.

Memory-like NK cells induced by viral infection or cytokine stimulation have been reported.[47,48] In many cases, such memory-like NK cells become antigen specific in their reactivation. Given that the majority of memory NK cells reside in the liver,[49] we speculate that memory NK cells may participate in the inflammatory process of PBC in an antigen-specific way. The potential role of memory NK cells in PBC in terms of their interaction with and modulation of T cells and acquired immune responses either directly or indirectly[50,51] should also be explored.

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ORIGINAL ARTICLE

Current status of deceased donor split liver transplantation in Japan

Seisuke Sakamoto · Mureo Kasahara · Yasuhiro Ogura · Yukihiro Inomata · Shinji Uemoto on behalf of the Japanese Liver Transplantation Society

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Abstract
Background The aim of this study was to analyze the outcomes of deceased donor split liver transplantation (LT) in Japan.
Methods Among 257 deceased donor LTs, 36 recipients underwent split LT until the end of 2014. The clinical data of donors and recipients were collected from the national organ transplant network and 11 liver transplant institutions, and the outcomes of split LT were evaluated.
Results Most of the whole livers were divided using the *ex-situ* splitting technique. Twelve livers were divided to left lateral segment/extended right lobe grafts and six livers to left lobe/right lobe grafts. The common underlying liver diseases consisted of graft failure in 11 patients, followed by acute liver failure in nine. Seventeen cases (48.6%) suffered from surgical complications, including biliary complications in six, all of which occurred in the cases receiving right-sided lobe grafts; intra-abdominal hemorrhage in five; and vascular complications in four. The overall graft 1-year and 3-year survival rates were 91.0% and 87.1%, respectively.
Conclusions An initial experience of deceased donor split LT in Japan shows acceptable outcomes despite the high incidence of surgical complications. Further advances in the development of the splitting technique are necessary to expand the application of split LT.

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Keywords Deceased donor · *In-situ/ex-situ* splitting technique · Split liver transplantation

Introduction

The number of deceased donor liver transplantation (DDLT) has gradually increased in Japan since the enforcement of a new law on organ transplantation in 2010. In contrast, living donor liver transplantation (LDLT) has been an established therapeutic option for patients with end-stage liver disease, and LDLT has been adopted as an optional life-saving procedure for the patients in urgent need of liver transplantation (LT) [1]. The concept of an LDLT program should be established, considering the donor safety as the first priority. According to the surveillance of outcomes in living donors from the Japanese Liver Transplantation Society (JLTS), approximately 8% of the donors suffered from complications related to liver donation, including death in one donor [2].

Split liver transplantation could be an excellent option for the expansion of the donor organ pool, even if only slightly, to overcome the challenge of limited graft resources, which largely depend on living donors. However, increased morbidity in recipients undergoing split LT has been reported, thus leading to the need for extreme caution when considering this procedure [3].

We herein retrospectively analyzed the initial outcomes of split LT in Japan to focus on the present issues related to this field.

Patients and methods

Among 257 DDLTs performed between February 1999 and December 2014 in Japan, 36 recipients (14.0%) underwent split LT. The deceased donor livers using split LT were divided to two hepatic grafts, consisting of 12 left lateral segments (LLS)/extended right lobes (ERL) for each recipient and six left lobes (LL) with middle hepatic vein (MHV)/right