0.37-0.76) were independently associated with the presence of severe fibrosis.

Comparison of Variables Associated with the Presence of Cirrhosis by Univariate and Multivariate Analysis. Variables associated with the presence of cirrhosis were assessed by univariate and multivariate analysis (Table 3). Age ($P = 0.0016$), AST ($P = 0.016$), GGT ($P = 0.0031$), bilirubin ($P < 0.0001$), α2-MG ($P = 0.019$), HA ($P < 0.0001$), TIMP1 ($P < 0.0001$), and AOL/DSA ($P < 0.0001$) were significantly higher in the cirrhosis group than in the no cirrhosis group. Albumin ($P < 0.0001$), cholinesterase ($P < 0.0001$), cholesterol ($P < 0.0001$), platelets ($P < 0.0001$), prothrombin time ($P = 0.0004$), and MAL/DSA ($P < 0.0001$) were significantly lower in the cirrhosis group than in the no cirrhosis group. Multivariate analysis showed that platelets (OR: 0.76, 95% CI: 0.58-0.99) and MAL/DSA (OR: 0.67, 95% CI: 0.49-0.90) were independently associated with the presence of cirrhosis.

Evaluation of the Two Glyco-Parameters AOL/DSA and MAL/DSA for Estimating the Progression of Liver Fibrosis. To assess the correlation of the two obtained glyco-parameters with the progression of fibrosis, we analyzed the data of triple lectins from HISCL measurements on the 183 CHC patients. The boxplots of AOL/DSA and MAL/DSA in relation to the fibrosis staging are shown in Fig. 1A,B, respectively. The AOL/DSA values gradually increased with the progression of fibrosis and Pearson's correlation efficient was R = 0.61. On the other hand, the MAL/DSA values gradually decreased with the progression of fibrosis and Pearson's correlation efficient was R = −0.69. Both parameters fitted the quantification of the progression of fibrosis from F2 to F4.

LecT-Hepa, Combined with Two Glyco-Parameters, Was Evaluated in the Diagnosis of Significant Fibrosis, Severe Fibrosis, and Cirrhosis. LecT-Hepa was calculated using two glyco-parameters (AOL/DSA and MAL/DSA). The boxplots of LecT-Hepa in relation to the fibrosis staging are shown in Fig. 2. The LecT-Hepa values gradually increased with the progression of fibrosis. Pearson's correlation coefficient between LecT-Hepa and liver fibrosis was very high (R = 0.72), and was superior to those for AOL/DSA (R = 0.61) and MAL/DSA (R = −0.69). We next examined AUC to characterize the diagnostic accuracy of LecT-Hepa at each stage of fibrosis, i.e., significant fibrosis (F2/F3/F4), severe fibrosis (F3/F4), and cirrhosis (F4). For the prediction of significant fibrosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) of the test were 0.802 (0.738-0.865), 59.6%, 89.9%, 85.7%, 66.7%, 5.89, and 0.45,

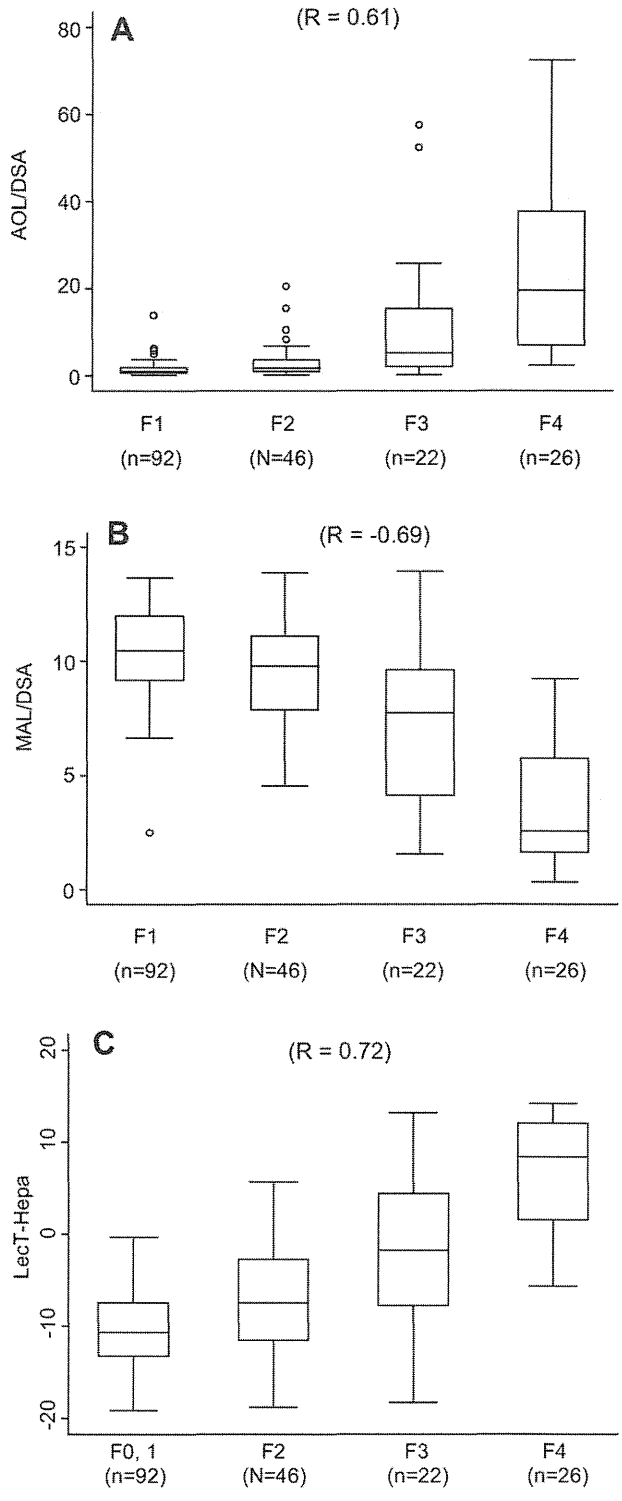
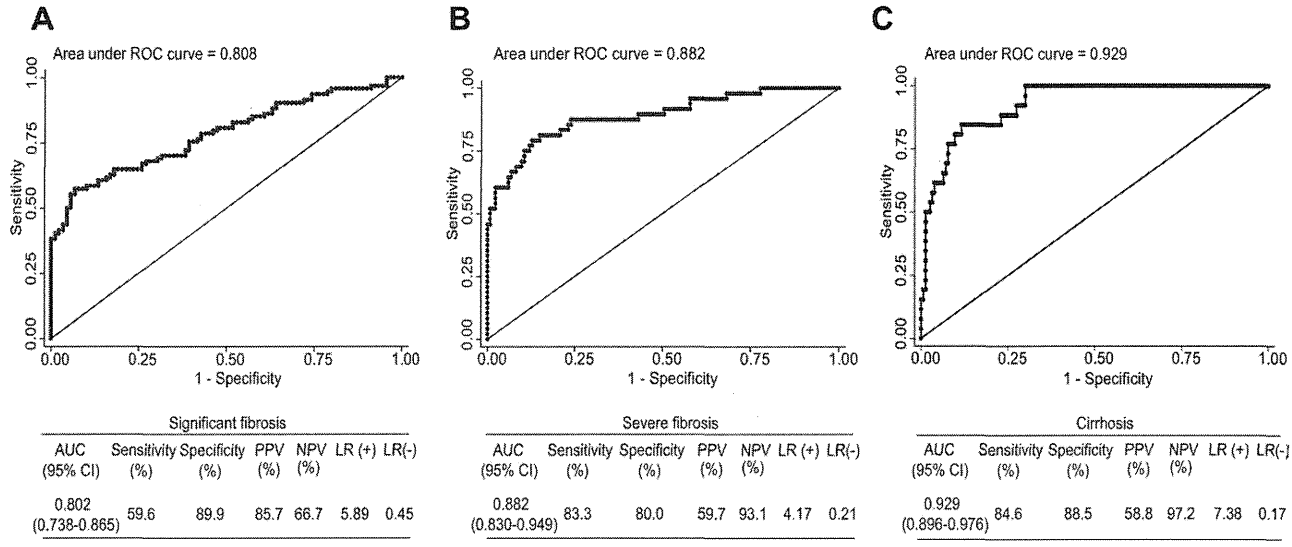


Fig. 1. Boxplot of (A) AOL/DSA, (B) MAL/DSA, and (C) LecT-Hepa in relation to the fibrosis score. The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the dots represent outliers. The line across the box indicates the median value. Correlation of AOL/DSA, MAL/DSA, and LecT-Hepa was measured by HISCL with the progression of liver fibrosis. R: Pearson's correlation coefficient.



Significant fibrosis

AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	5.89	0.45

Severe fibrosis

AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.882 (0.830-0.949)	83.3	80.0	59.7	93.1	4.17	0.21

Cirrhosis

AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR (+)	LR(-)
0.929 (0.896-0.976)	84.6	88.5	58.8	97.2	7.38	0.17

Fig. 2. ROC curves of LecT-Hepa to distinguish between significant fibrosis and no significant fibrosis in patients with chronic hepatitis C (A); severe fibrosis and no severe fibrosis (B); cirrhosis and no cirrhosis (C). AUC: area under the receiver operating characteristic curve; PPV: positive predictive values; NPV: negative predictive values; LR (+): positive likelihood ratio; LR (−): negative likelihood ratio.

respectively (Fig. 3A). For the prediction of severe fibrosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) were 0.882, 83.3%, 80.0%, 59.7%, 93.1%, 4.17, and 0.21, respectively (Fig. 3B). For the prediction of cirrhosis, AUC (95% CI), sensitivity, specificity, PPV, NPV, LR (+), and LR (−) were 0.929 (0.896-0.976), 84.6%, 88.5%, 58.8%, 97.2%, 7.38, and 0.17, respectively (Fig. 3C).

Comparison of AUC, Sensitivity, Specificity, PPV, and NPV for Predicting the Diagnosis of Significant Fibrosis, Severe Fibrosis, and Cirrhosis. ROC curves of LecT-Hepa, HA, TIMP1, platelets, APRI, Forns index, Fib-4 index, and Zeng's score for predicting significant fibrosis, severe fibrosis, and cirrhosis were plotted, as shown in Fig. 3A-C. The AUC of LecT-Hepa for predicting significant fibrosis (0.802) was superior to HA (0.756), TIMP1 (0.697), platelets (0.729), APRI (0.777), Fib-4 index (0.747), Forns index (0.783), and Zeng's score (0.791). For predicting severe fibrosis, AUC of LecT-Hepa (0.882) was superior to HA (0.839), TIMP1 (0.753), platelet count (0.821), APRI (0.840), Fib-4 index (0.811), Forns index (0.861), and Zeng's score (0.863). For predicting cirrhosis, AUC of LecT-Hepa (0.929) was superior to HA (0.866), TIMP1 (0.783), platelets (0.851), APRI (0.787), Fib-4 index (0.856), Forns index (0.887), and Zeng's score (0.853). Sensitivity, specificity, PPV, and NPV by eight noninvasive tests and markers are shown in Table 4. In general, indicators of LecT-Hepa were superior to other noninvasive tests and markers. Specificity and PPV used to distinguish significant fibrosis in LecT-Hepa were superior to those in other tests and markers, although sensitivity and NPV by LecT-Hepa (59.6% and 66.7%, respectively) to distinguish significant fibrosis were inferior to those in other tests and markers. When distinguishing severe fibrosis, the categories of sensitivity (83.3%), specificity (80.0%), PPV (59.7%), and NPV (93.1%) for LecT-Hepa were superior to those in other tests and markers, except for specificity (82.2%) and PPV (61.0%) in HA. When distinguishing cirrhosis, the categories of sensitivity (84.6%), specificity (88.5%), PPV (58.8%), and NPV (97.2%) in LecT-Hepa were superior to those in other tests and markers, except for sensitivity by HA (88.5%), Forns index (84.6%), and Zeng's score (92.3%) and NPV by Zeng's score (98.3%).

Discussion

Our results showed that the LecT-Hepa test, calculated by combining two glyco-parameters (AOL/DSA and MAL/DSA), had higher sensitivity and specificity for diagnosing severe fibrosis and cirrhosis compared to other noninvasive tests and markers for these conditions. The new glyco-marker we have developed is based on the glyco-alteration on the AGP, which is mainly synthesized in the liver. AGP has been considered one of the best candidates for glyco-markers in liver fibrosis or HCC. This is because it is a well-characterized glycoprotein with five highly branched, complex-type *N*-glycans, whose alteration (e.g., desialylation, increased branching, and increased fucosylation) occurs during the progression of liver fibrosis and carcinogenesis.[24] It has already been reported that an

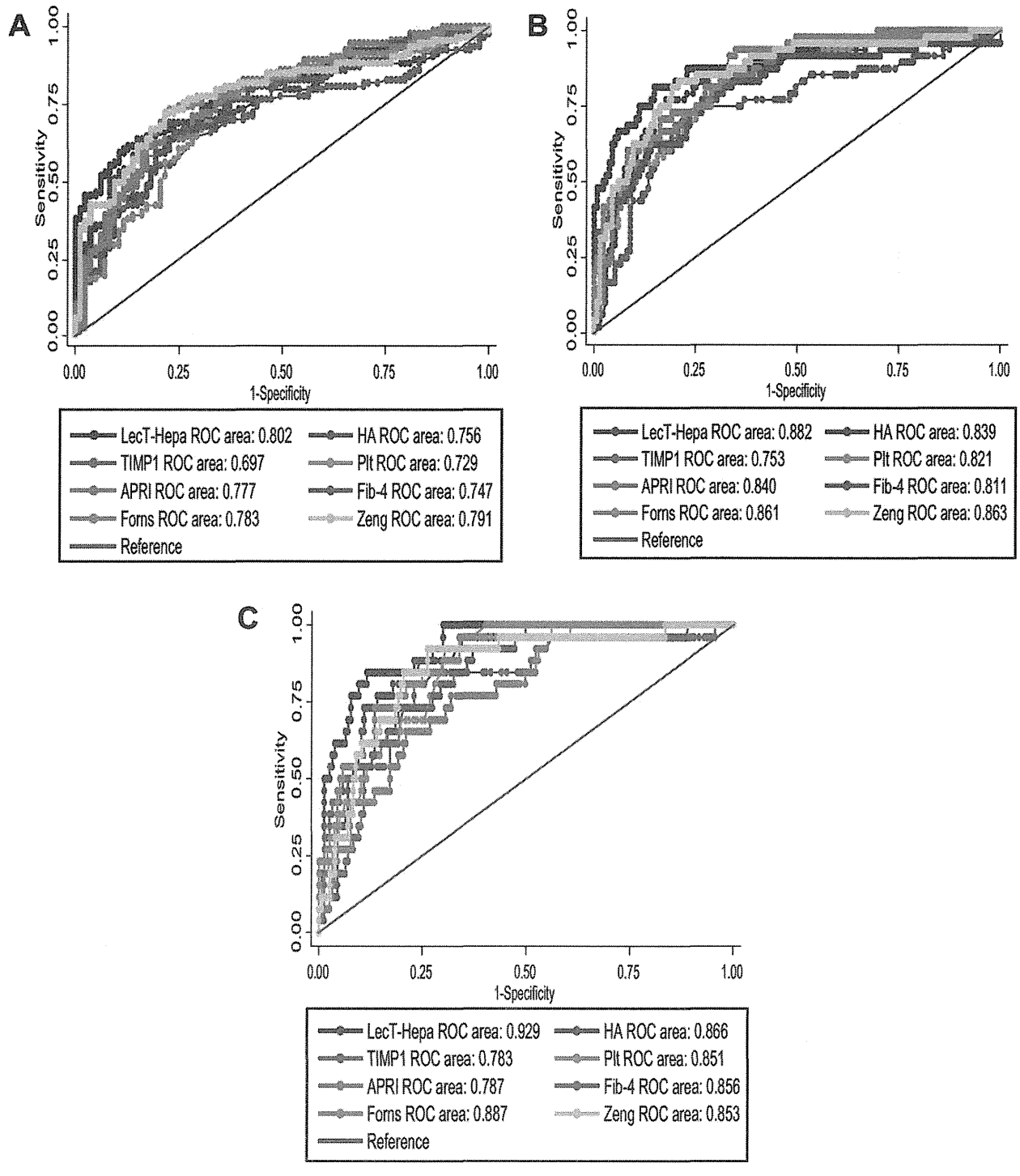


Fig. 3. Comparison of ROC curves in the performance of LecT-Hepa, HA, TIMP1, Plt, APRI, Fib-4 Index, Forns index, Zeng's score for the diagnosis of significant fibrosis (A), severe fibrosis (B), and cirrhosis (C). ROC: receiver operating characteristic curve; TIMP1: tissue inhibitors of metalloproteinases 1; Plt: platelet count; HA: hyaluronic acid.

increased degree of fucosylation was detected in cirrhosis patients using a fucose-binding lectin (AAL)-antibody sandwich ELISA and an automated analyzer.[24] The detection of asialo-AGP using lactosamine-recognition lectin RCA120 has also been reported as an alternative method for finding cirrhosis.[25] Meanwhile, we detected many other aspects of glyco-alteration of AGP using a multiplex sandwich immunoassay with a 43-lectin microarray,[26] resulting in the selection of three lectins—MAL, AOL, and DSA—to serve, collectively, as a fibrosis indicator and a signal normalizer.[14] Since two glyco-parameters (AOL/DSA and MAL/

Table 4. Diagnostic Performance of Biochemical Markers and Scores by Stage of Fibrosis

	No Significant Fibrosis (F0-1) vs. Significant Fibrosis (F2-4)					No Severe Fibrosis (F0-2) vs. Severe Fibrosis (F3-4)					No Cirrhosis (F0-3) vs. Cirrhosis (F4)				
	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)	Se (%)	Sp (%)	PPV (%)	NPV (%)
LecT-Hepa	0.802 (0.738-0.865)	59.6	89.9	85.7	66.7	0.882 (0.830-0.949)	83.3	80	59.7	93.1	0.929 (0.896-0.976)	84.6	88.5	58.8	97.2
HA	0.756 (0.684-0.827)	68.1	78.7	77.8	69.6	0.839 (0.771-0.908)	77.1	82.2	61	90.3	0.866 (0.790-0.942)	88.5	75.8	37.3	96.8
TIMP1	0.697 (0.619-0.774)	65.9	71.9	70.4	60.7	0.753 (0.665-0.841)	75	76.3	53	88.9	0.783 (0.710-0.887)	80.8	74.5	27.8	94.6
Platelets	0.729 (0.656-0.803)	78.7	61.9	68.5	73.5	0.821 (0.751-0.891)	81.3	70.4	49.4	91.3	0.851 (0.785-0.918)	84.6	70.7	32.3	95.8
APRI	0.777 (0.709-0.844)	71.3	71.9	72.2	68.8	0.840 (0.780-0.900)	81.3	72.6	50.6	91.5	0.787 (0.703-0.871)	76.9	68.2	27.9	93.9
Fib-4	0.747 (0.671-0.818)	65.9	76.4	74.7	68	0.811 (0.733-0.889)	77.1	73.3	50	89.2	0.856 (0.788-0.924)	73.1	80.9	37.5	94.1
Forns	0.783 (0.716-0.852)	73.4	77.5	77.5	73.4	0.861 (0.802-0.920)	81.3	71.1	50	91.4	0.887 (0.831-0.943)	84.6	75.2	36.1	96.7
Zeng	0.791 (0.723-0.858)	82.9	70.7	75	79.7	0.863 (0.799-0.925)	81.3	79.8	59.5	92.8	0.853 (0.783-0.933)	92.3	73.9	36.9	98.3

AUC, area under the ROC curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive values; NPV, negative predictive values.

DSA) on AGP are normalized by an internal standard lectin (DSA), LecT-Hepa is not influenced by the amount of AGP. We confirmed that the use of this lectin set was statistically superior to the previously selected lectins (AAL and RCA120).

This triplex-sandwich immunoassay employing DSA/MAL/AOL lectins and an anti-AGP antibody from the lectin microarray has already been converted to a fully automated immunoassay analyzer (HISCL-2000i) for clinical use.[15] Pretreatment requires 3 hours, and quantifying the two glyco-parameters for the LecT-Hepa to use this automated analyzer takes 17 minutes. Currently, we can obtain data from LecT-Hepa to predict liver fibrosis on the same day of blood sample collection. This simple and reliable glycomarker may be suitable for clinical use, and may substitute for liver biopsy in some cases.

We are confident that our study samples are representative of most patients. The AUC scores for distinguishing significant fibrosis, severe fibrosis, and cirrhosis by APRI, HA, Fib-4 index, Forns index, and Zeng's score were not significantly different from those in previous studies.[11,27,28] Every serum sample in this study was obtained from a patient immediately before or no more than 2 months after liver biopsy. As many serum samples as possible were collected from each liver center to eliminate a selection bias in any center. Since we could not perform liver biopsy on the patients who had a tendency to develop hemorrhages, fewer samples of severe fibrosis and cirrhosis were collected than those of milder fibrosis. In fact, the population of fibrosis staging in this study was similar to that of a previous, large prospective study evaluating noninvasive fibrosis markers.[29] In addition, we did not include patients with obvious decompensated cirrhosis. This is because inclusion of patients with severe liver disease would have artificially improved the predictive values of the logistic function. On the other hand, we included many patients with mild histological features (48.6% with F0-1). Sampling variation poses potential difficulties, especially in the early stages of disease, when fibrosis might be unevenly distributed.

There are several advantages in using reliable noninvasive markers for assessing liver fibrosis. First, they can be used to accurately determine the appropriate time for initiating IFN treatment in CHC patients. These markers can also help monitor and assess the therapeutic efficacy of IFN treatment in improving liver function in cases of liver fibrosis and cirrhosis. Finally, these markers will be essential in the development of new, antifibrotic treatments. Recently, many directed or targeted therapies against liver fibrosis,

such as anti-transforming growth factor beta and anti-tumor necrosis factor alpha compounds have been developed.[30,31] To evaluate these new drugs, reliable and simple noninvasive fibrosis markers are needed. LecT-Hepa appears to be one of the most prominent candidates to serve as a marker for developing antifibrotic drugs.

In conclusion, both glyco-parameters (AOL/DSA and MAL/DSA) using lectins in a bedside, clinical chemical analyzer succeeded in the quantification of the progression of liver fibrosis. Using LecT-Hepa, the combination score of both AOL/DSA and MAL/DSA is a reliable method for determining fibrosis staging and can be a good substitute for liver biopsy.

Acknowledgment: We thank K. Saito, S. Unno, T. Fukuda, and M. Sogabe (AIST) for technical assistance. We also thank C. Tsuruno, S. Nagai, and Y. Takahama (Sysmex Co.) for critical discussion.

References

1. Yano M, Kumada H, Kage M, Ikeda K, Shimamatsu K, Inoue O, et al. The long-term pathological evolution of chronic hepatitis C. HEPATOLOGY 1996;23:1334-1340.
2. Forns X, Ampurdanes S, Sanchez-Tapias JM, Guilera M, Sans M, Sanchez-Fueyo A, et al. Long-term follow-up of chronic hepatitis C in patients diagnosed at a tertiary-care center. J Hepatol 2001;35:265-271.
3. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. HEPATOLOGY 2009;49: 1335-1374.
4. Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). HEPATOLOGY 2000;32:477-481.
5. Castera L, Negre I, Samii K, Buffet C. Pain experienced during percutaneous liver biopsy. HEPATOLOGY 1999;30:1529-1530.
6. Bravo AA, Sheth SG, Chopra S. Liver biopsy. N Engl J Med 2001; 344:495-500.
7. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. HEPATOLOGY 2003;38:518-526.
8. Regev A, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, et al. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. Am J Gastroenterol 2002;97: 2614-2618.
9. Oberti F, Valsesia E, Pilette C, Rousselet MC, Bedossa P, Aube C, et al. Noninvasive diagnosis of hepatic fibrosis or cirrhosis. Gastroenterology 1997;113:1609-1616.
10. Afdhal NH, Nunes D. Evaluation of liver fibrosis: a concise review. Am J Gastroenterol 2004;99:1160-1174.
11. Cales P, Oberti F, Michalak S, Hubert-Fouchard I, Rousselet MC, Konate A, et al. A novel panel of blood markers to assess the degree of liver fibrosis. HEPATOLOGY 2005;42:1373-1381.
12. Ziol M, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. HEPATOLOGY 2005;41:48-54.
13. Castera L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology 2005;128:343-350.
14. Kuno A, Ikehara Y, Tanaka Y, Angata T, Unno S, Sogabe M, et al. Multilectin assay for detecting fibrosis-specific glyco-alteration by means of lectin microarray. Clin Chem 2011;57:48-56.
15. Kuno A, Ikehara Y, Tanaka Y, Saito K, Ito K, Tsuruno C, et al. LecT-Hepa: a triplex lectin-antibody sandwich immunoassay for estimating the progression dynamics of liver fibrosis assisted by a bedside clinical chemistry analyzer and an automated pretreatment machine. Clin Chim Acta 2011;412:1767-1772.
16. Matsuda A, Kuno A, Kawamoto T, Matsuzaki H, Irimura T, Ikehara Y, et al. Wisteria floribunda agglutinin-positive mucin 1 is a sensitive biliary marker for human cholangiocarcinoma. HEPATOLOGY 2010;52:174-182.
17. Ohkura T, Hada T, Higashino K, Ohue T, Kochibe N, Koide N, et al. Increase of fucosylated serum cholinesterase in relation to high risk groups for hepatocellular carcinomas. Cancer Res 1994;54:55-61.
18. Turner GA. N-glycosylation of serum proteins in disease and its investigation using lectins. Clin Chim Acta 1992;208:149-171.
19. Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, et al. Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. N Engl J Med 1993;328:1802-1806.
20. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. The French METAVIR Cooperative Study Group. HEPATOLOGY 1994;20:15-20.
21. Zeng MD, Lu LG, Mao YM, Qiu DK, Li JQ, Wan MB, et al. Prediction of significant fibrosis in HBeAg-positive patients with chronic hepatitis B by a noninvasive model. HEPATOLOGY 2005;42:1437-1445.
22. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. HEPATOLOGY 2006;43: 1317-1325.
23. Leroy V, Halfon P, Bacq Y, Boursier J, Rousselet MC, Bourliere M, et al. Diagnostic accuracy, reproducibility and robustness of fibrosis blood tests in chronic hepatitis C: a meta-analysis with individual data. Clin Biochem 2008;41:1368-1376.
24. Ryden I, Pahlsson P, Lindgren S. Diagnostic accuracy of alpha (1)-acid glycoprotein fucosylation for liver cirrhosis in patients undergoing hepatic biopsy. Clin Chem 2002;48:2195-2201.
25. Kim KA, Lee EY, Kang JH, Lee HG, Kim JW, Kwon DH, et al. Diagnostic accuracy of serum asialo-alpha1-acid glycoprotein concentration for the differential diagnosis of liver cirrhosis and hepatocellular carcinoma. Clin Chim Acta 2006;369:46-51.
26. Kuno A, Uchiyama N, Koseki-Kuno S, Ebe Y, Takashima S, Yamada M, et al. Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. Nat Methods 2005;2:851-856.
27. Forns X, Ampurdanes S, Llovet JM, Aponte J, Quinto L, Martinez-Bauer E, et al. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. HEPATOLOGY 2002;36: 986-992.
28. Bottero J, Lacombe K, Guechot J, Serfaty L, Miailhes P, Bonnard P, et al. Performance of 11 biomarkers for liver fibrosis assessment in HIV/HBV co-infected patients. J Hepatol 2009;50:1074-1083.
29. Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet 2001;357:1069-1075.
30. Yata Y, Gotwals P, Koteliansky V, Rockey DC. Dose-dependent inhibition of hepatic fibrosis in mice by a TGF-beta soluble receptor: implications for antifibrotic therapy. HEPATOLOGY 2002;35:1022-1030.
31. Akriviadis E, Botla R, Briggs W, Han S, Reynolds T, Shakil O. Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: a double-blind, placebo-controlled trial. Gastroenterology 2000;119: 1637-1648.

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Human Blood Dendritic Cell Antigen 3 (BDCA3)$^+$ Dendritic Cells Are a Potent Producer of Interferon-λ in Response to Hepatitis C Virus

Sachiyo Yoshio,[1] Tatsuya Kanto,[1] Shoko Kuroda,[1] Tokuhiro Matsubara,[1] Koyo Higashitani,[1] Naruyasu Kakita,[1] Hisashi Ishida,[1] Naoki Hiramatsu,[1] Hiroaki Nagano,[2] Masaya Sugiyama,[3] Kazumoto Murata,[3] Takasuke Fukuhara,[4] Yoshiharu Matsuura,[4] Norio Hayashi,[5] Masashi Mizokami,[3] and Tetsuo Takehara[1]

The polymorphisms in the interleukin (*IL)-28B* (interferon-lambda [IFN]-λ3) gene are strongly associated with the efficacy of hepatitis C virus (HCV) clearance. Dendritic cells (DCs) sense HCV and produce IFNs, thereby playing some cooperative roles with HCV-infected hepatocytes in the induction of interferon-stimulated genes (ISGs). Blood dendritic cell antigen 3 (BDCA3)$^+$ DCs were discovered as a producer of IFN-λ upon Toll-like receptor 3 (TLR3) stimulation. We thus aimed to clarify the roles of BDCA3$^+$ DCs in anti-HCV innate immunity. Seventy healthy subjects and 20 patients with liver tumors were enrolled. BDCA3$^+$ DCs, in comparison with plasmacytoid DCs and myeloid DCs, were stimulated with TLR agonists, cell-cultured HCV (HCVcc), or Huh7.5.1 cells transfected with HCV/JFH-1. BDCA3$^+$ DCs were treated with anti-CD81 antibody, inhibitors of endosome acidification, TIR-domain-containing adapter-inducing interferon-β (TRIF)-specific inhibitor, or ultraviolet-irradiated HCVcc. The amounts of IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B were quantified by subtype-specific enzyme-linked immunosorbent assay (ELISA). The frequency of BDCA3$^+$ DCs in peripheral blood mononuclear cell (PBMC) was extremely low but higher in the liver. BDCA3$^+$ DCs recovered from PBMC or the liver released large amounts of IFN-λs, when stimulated with HCVcc or HCV-transfected Huh7.5.1. BDCA3$^+$ DCs were able to induce ISGs in the coexisting JFH-1-positive Huh7.5.1 cells. The treatments of BDCA3$^+$ DCs with anti-CD81 antibody, cloroquine, or bafilomycin A1 reduced HCVcc-induced IL-28B release, whereas BDCA3$^+$ DCs comparably produced IL-28B upon replication-defective HCVcc. The TRIF-specific inhibitor reduced IL-28B release from HCVcc-stimulated BDCA3$^+$ DCs. In response to HCVcc or JFH-1-Huh7.5.1, BDCA3$^+$ DCs in healthy subjects with IL-28B major (rs8099917, TT) released more IL-28B than those with IL-28B minor genotype (TG). *Conclusion*: Human BDCA3$^+$ DCs, having a tendency to accumulate in the liver, recognize HCV in a CD81-, endosome-, and TRIF-dependent manner and produce substantial amounts of IL-28B/IFN-λ3, the ability of which is superior in subjects with IL-28B major genotype. (HEPATOLOGY 2012;00:000–000)

Hepatitis C virus (HCV) infection is one of the most serious health problems in the world. More than 170 million people are chronically infected with HCV and are at high risk of developing liver cirrhosis and hepatocellular carcinoma. Genome-wide association studies have successfully identified the genetic polymorphisms (single nucleotide polymorphisms, SNPs) upstream of the promoter region of the

Abbreviations: Ab, antibody; HCV, hepatitis C virus; HCVcc, cell-cultured hepatitis C virus; HSV, herpes simplex virus; IHL, intrahepatic lymphocyte; INF-λ, interferon-lambda; IRF, interferon regulatory factor; ISGs, interferon-stimulated genes; JEV, Japanese encephalitis virus; Lin, lineage; mDC, myeloid DC; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid DC; Poly IC, polyinosine-polycytidylic acid; RIG-I, retinoic acid-inducible gene-I; SNPs, single nucleotide polymorphisms; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

From the [1]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan; [2]Department of Surgery, Osaka University Graduate School of Medicine, Osaka, Japan; [3]Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, Japan; [4]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; [5]Kansai Rosai Hospital, Hyogo, Japan.

Received July 2, 2012; accepted November 13, 2012.

Supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and a Grant-In-Aid from the Ministry of Health, Labor, and Welfare of Japan.

interleukin (IL)-28B / interferon-lambda 3 (IFN-λ3) gene, which are strongly associated with the efficacy of pegylated interferon-α (PEG-IFN-α) and ribavirin therapy or spontaneous HCV clearance.[1-4]

IFN-λs, or type III IFNs, comprise a family of highly homologous molecules consisting of IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B). In clear contrast to type I IFNs, they are released from relatively restricted types of cells, such as hepatocytes, intestinal epithelial cells, or dendritic cells (DCs). Also, the cells that express heterodimeric IFN-λ receptors (IFN-λR1 and IL-10R2) are restricted to cells of epithelial origin, hepatocytes, or DCs.[5] Such limited profiles of cells expressing IFN-λs and their receptors define the biological uniqueness of IFN-λs. It has been shown that IFN-λs convey anti-HCV activity by inducing various interferon-stimulated genes (ISGs),[5] the profiles of which were overlapped but others were distinct from those induced by IFN-α/β. Some investigators showed that the expression of IL-28 in PBMC was higher in subjects with IL-28B major than those with minor; however, the levels of IL-28 transcripts in liver tissue were comparable regardless of IL-28B genotype.[2,6]

At the primary exposure to hosts, HCV maintains high replicative levels in the infected liver, resulting in the induction of IFNs and ISGs. In a case of successful HCV eradication, it is postulated that IFN-α/β and IFN-λ cooperatively induce antiviral ISGs in HCV-infected hepatocytes. It is of particular interest that, in primary human hepatocytes or chimpanzee liver, IFN-λs, but not type I IFNs, are primarily induced after HCV inoculation, the degree of which is closely correlated with the levels of ISGs.[7] These results suggest that hepatic IFN-λ could be a principal driver of ISG induction in response to HCV infection. Nevertheless, the possibility remains that DCs, as a prominent IFN producer in the liver, play significant roles in inducing hepatic ISGs and thereby suppressing HCV replication.

DCs, as immune sentinels, sense specific genomic and/or structural components of pathogens with various pattern recognition receptors and eventually release IFNs and inflammatory cytokines.[8] In general, DCs migrate to the organ where inflammation or cellular apoptosis occurs and alter their function in order to alleviate or exacerbate the disease conditions. Therefore, the phenotypes and/or capacity of liver DCs are deemed to be influenced in the inflamed liver. In humans, the existence of phenotypically and functionally distinct DC subsets has been reported: myeloid DC (mDC) and plasmacytoid DC (pDC).[9] Myeloid DCs predominantly produce IL-12 or tumor necrosis factor alpha (TNF-α) following proinflammatory stimuli, while pDCs release considerable amounts of type I IFNs upon virus infection.[9] The other type of mDCs, mDC2 or BDCA3$^+$(CD141) DCs, have been drawing much attention recently, since human BDCA3$^+$ DCs are reported to be a counterpart of murine CD8a$^+$ DCs.[10] Of particular interest is the report that BDCA3$^+$ DCs have a potent capacity of releasing IFN-λ in response to Toll-like receptor 3 (TLR3) agonist.[11] However, it is still largely unknown whether human BDCA3$^+$ DCs are able to respond to HCV.

Taking these reports into consideration, we hypothesized that human BDCA3$^+$ DCs, as a producer of IFN-λs, have crucial roles in anti-HCV innate immunity. We thus tried to clarify the potential of BDCA3$^+$ DCs in producing type III IFNs by using cell-cultured HCV (HCVcc) or hepatoma cells harboring HCV as stimuli. Our findings show that BDCA3$^+$ DCs are quite a unique DC subset, characterized by a potent and specialized ability to secrete IFN-λs in response to HCV. The ability of BDCA3$^+$ DCs to release IL-28B upon HCV is superior in subjects with IL-28B major (rs8099917, TT) to those with minor (TG or GG) genotype, suggesting that BDCA3$^+$ DCs are one of the key players in IFN-λ-mediated innate immunity.

Patients and Methods

Subjects. This study enrolled 70 healthy volunteers (male/female: 61/9) (age: mean ± standard deviation [SD], 37.3 ± 7.8 years) and 20 patients who underwent surgical resection of liver tumors at Osaka University Hospital (Supporting Table 1). The study was approved by the Ethical Committee of Osaka University Graduate School of Medicine. Written informed consent was obtained from all of them. All healthy volunteers were negative for HCV, hepatitis B virus (HBV), and human immunodeficiency virus (HIV) and had no apparent history of liver, autoimmune, or malignant diseases.

Address reprint requests to: Tatsuya Kanto, M.D., Ph.D., Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, 565-0871 Japan. E-mail: kantot@gh.med.osaka-u.ac.jp; fax: +81-6-6879-3629.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.26182

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Reagents. The specifications of all antibodies used for FACS or cell sorting TLR-specific synthetic agonists, pharmacological reagents, and inhibitory peptides are listed in the Supporting Materials.

Separation of DCs from PBMC or Intrahepatic Lymphocytes. We collected 400 mL of blood from each healthy volunteer and processed them for PBMCs. Noncancerous liver tissues were obtained from patients who underwent resection of liver tumors (Supporting Table 1). For the collection of intrahepatic lymphocytes (IHLs), liver tissues were washed thoroughly with phosphate-buffered saline to remove the peripheral blood adhering to the tissue and ground gently. After Lin-negative ($CD3^-$, $CD14^-$, $CD19^-$, and $CD56^-$) cells were obtained by the MACS system, each DC subset with the defined phenotype was sorted separately under FACS Aria (BD). The purity was more than 98%, as assessed by FACS Canto II (BD). Sorted DCs were cultured at 2.5×10^4/well on 96-well culture plates.

Immunofluorescence Staining of Human Liver Tissue. Tissue specimens were obtained from surgical resections of noncancerous liver from the patients as described above. Briefly, the 5-mm sections were incubated with the following antibodies: mouse biotinylated antihuman BDCA3 antibody (Miltenyi-Biotec), and mouse antihuman CLEC9A antibody (Biolegend) and subsequently with secondary goat antirabbit Alexa Fluor488 or goat antimouse Alexa Fluor594 (Invitrogen, Molecular Probes) antibodies. Cell nuclei were counterstained with Dapi-Fluoromount-GTM (Southern Biotech, Birmingham, AL). The stained tissues were analyzed by fluorescence microscopy (Model BZ-9000; Keyence, Osaka, Japan).

Cells and Viruses. The *in vitro* transcribed RNA of the JFH-1 strain of HCV was introduced into FT3-7 cells[12] or Huh7.5.1 cells. The stocks of HCVcc were generated by concentration of the medium from JFH-1-infected FT3-7 cells. The virus titers were determined by focus forming assay.[13] The control medium was generated by concentration of the medium from HCV-uninfected FT3-7 cells. Infectious JEVs were generated from the expression plasmid (pMWJEATG1) as reported.[14] HSV (KOS) was a generous gift from Dr. K. Ueda (Osaka University). Huh7.5.1 cells transduced with HCV JFH-1 strain was used for the coculture with DCs. The transcripts of ISGs in Huh7.5.1 were examined by reverse-transcription polymerase chain reaction (RT-PCR) methods using gene-specific primers and probes (Applied Biosystems, Foster City, CA).

Secretion Assays. IL-28B/IFN-λ3 was quantified by a newly developed chemiluminescence enzyme immunoassay (CLEIA) system.[15] IL-29/IFN-λ1, IL-28A/IFN-λ2, and IFN-β were assayed by commercially available enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, R&D, and PBL, respectively). IFN-α was measured by cytometric beads array kits (BD) according to the manufacturer's instructions.

Statistical Analysis. The differences between two groups were assessed by the Mann-Whitney nonparametric *U* test. Multiple comparisons between more than two groups were analyzed by the Kruskal-Wallis nonparametric test. Paired *t* tests were used to compare differences in paired samples. All the analyses were performed using GraphPad Prism software (San Diego, CA).

Results

Human BDCA3$^+$ DCs Are Phenotypically Distinct from pDCs and mDCs. We defined BDCA3$^+$ DCs as $Lin^-HLA\text{-}DR^+BDCA3^{high+}$ cells (Fig. 1A, left, middle), and pDCs and mDCs by the patterns of CD11c and CD123 expressions (Fig. 1A, right). The level of CD86 on pDCs or mDCs is comparatively higher than those on BDCA3$^+$ DCs (Fig. 1B). The expression of CD81 is higher on BDCA3$^+$ DCs than on pDCs and mDCs (Fig. 1B, Supporting Fig. S1). CLEC9A, a member of C-type lectin, is expressed specifically on BDCA3$^+$ DCs as reported elsewhere,[16] but not on pDCs and mDCs (Fig. 1B).

Liver BDCA3$^+$ DCs Are More Mature than the Counterparts in the Periphery. BDCA3$^+$ DCs in infiltrated hepatic lymphocytes (IHLs) are all positive for CLEC9A, but liver pDCs or mDCs are not (data not shown). The levels of CD40, CD80, CD83, and CD86 on liver BDCA3$^+$ DCs are higher than those on the peripheral counterparts, suggesting that BDCA3$^+$ DCs are more mature in the liver compared to those in the periphery (Fig. 1C).

In order to confirm that BDCA3$^+$ DCs are localized in the liver, we stained the cells with immunofluorescence antibodies (Abs) in noncancerous liver tissues. Liver BDCA3$^+$ DCs were defined as BDCA3$^+$ CLEC9A$^+$ cells (Fig. 1D). Most of the cells were found near the vascular compartment or in sinusoid or the space of Disse of the liver tissue.

BDCA3$^+$ DCs Are Scarce in PBMCs but More Abundant in the Liver. The percentages of BDCA3$^+$ DCs in PBMCs were much lower than those of the other DC subsets (BDCA3$^+$ DCs, pDCs and mDCs, mean $\pm$ SD [%], 0.054 ± 0.044, 0.27 ± 0.21 and 1.30 ± 0.65) (Fig. 2A). The percentages of BDCA3$^+$ DCs in IHLs were lower than those of the others (BDCA3$^+$ DCs, pDCs, and mDCs, mean $\pm$ SD [%],

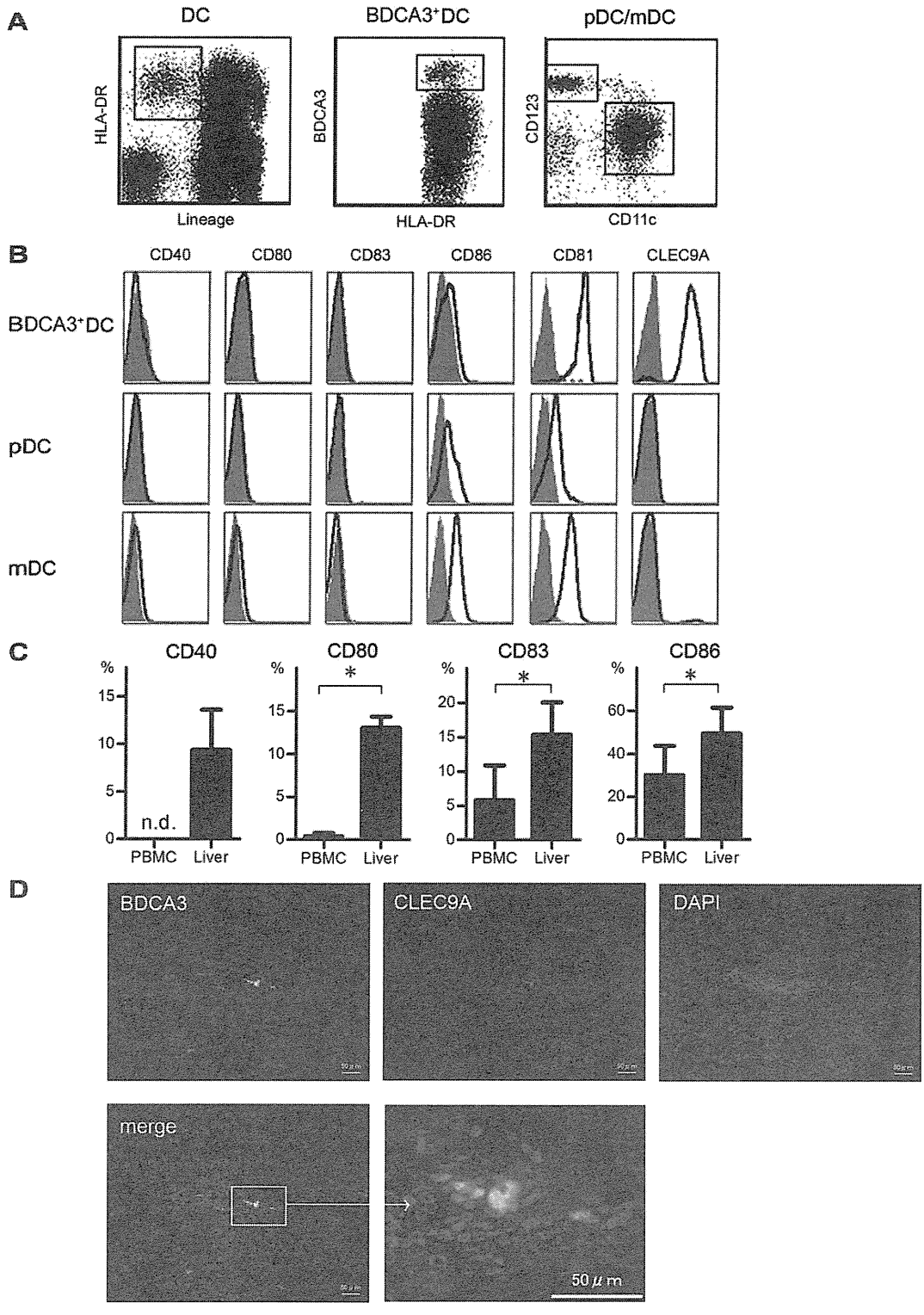


Fig. 1. Identification and phenotypic analyses of peripheral blood and intrahepatic BDCA3$^+$ DCs. (A) We defined BDCA3$^+$ DCs as Lineage$^-$HLA-DR$^+$BDCA3^{high+} cells (middle), pDCs as Lineage$^-$HLA-DR$^+$CD11c$^-$CD123^{high+} cells, and mDCs as Lineage$^-$HLA-DR$^+$CD11c$^+$CD123^{low+} cells (right). (B) The expressions of CD40, CD80, CD83, CD86, CD81, and CLEC9A on each DC subset in peripheral blood are shown. Representative results of five donors are shown in the histograms. Filled gray histograms depict data with isotype Abs, and open black ones are those with specific Abs. (C) The expressions of costimulatory molecules on BDCA3$^+$ DCs were compared between in PBMCs and in the liver. The results are shown as the percentage of positive cells. Results are the mean ± SEM from four independent experiments. *$P < 0.05$ by paired t test. (D) The staining for BDCA3 (green), CLEC9A (red) identifies BDCA3$^+$ DCs (merge, BDCA3$^+$CLEC9A$^+$) in human liver tissues. Representative results of the noncancerous liver samples are shown. BDCA, blood dendritic cell antigen; pDC, plasmacytoid DC; mDC, myeloid DC; CLEC9A, C-type lectin 9A.

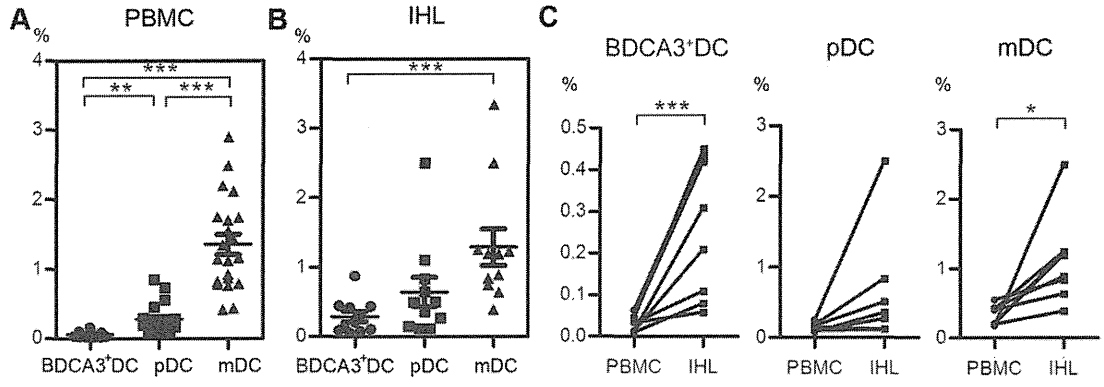


Fig. 2. Analysis of frequency of DC subsets in the peripheral blood and in the liver. Frequencies of BDCA3$^+$ DCs, pDCs, and mDCs in PBMCs (21 healthy subjects) (A) or in the intrahepatic lymphocytes (IHLs) (11 patients who had undergone surgical resection of tumors) (B) are shown. Horizontal bars depict the mean ± SD. $^{**}P < 0.005$; $^{***}P < 0.0005$ by Kruskal-Wallis test. (C) The paired comparisons of the frequencies of DC subsets between in PBMCs and in IHLs. the results of eight patients whose PBMCs and IHLs were obtained simultaneously are shown. $^{*}P < 0.05$; $^{***}P < 0.0005$ by paired *t* test. IHLs, intrahepatic lymphocytes; pDC and mDC, see Fig. 1.

0.29 ± 0.25, 0.65 ± 0.69 and 1.2 ± 0.94) (Fig. 2B). The percentages of BDCA3$^+$ DCs in the IHLs were significantly higher than those in PBMCs from relevant donors (Fig. 2C). Such relative abundance of BDCA3$^+$ DCs in the liver over that in the periphery was observed regardless of the etiology of the liver disease (Supporting Table 1).

BDCA3$^+$ DCs Produce a Large Amount of IFN-λs upon Poly IC Stimulation. We compared DC subsets for their abilities to produce IL-29/IFN-λ1, IL-28A/IFN-λ2, IL-28B/IFN-λ3, IFN-β, and IFN-α in response to TLR agonists. Approximately 4.0×10^4 of BDCA3$^+$ DCs were recoverable from 400 mL of donated blood from healthy volunteers. We fixed the number of DCs at 2.5×10^4 cells/100 mL for comparison in the following experiments.

BDCA3$^+$ DCs have been reported to express mRNA for TLR1, 2, 3, 6, 8, and 10.[17] First, we quantified IL-28B/IFN-λ3 as a representative for IFN-λs after stimulation of BDCA3$^+$ DCs with relevant TLR agonists. We confirmed that BDCA3$^+$ DCs released IL-28B robustly in response to TLR3 agonist/poly IC but not to other TLR agonists (Fig. S2). In contrast, pDCs produced IL-28B in response to TLR9 agonist/CpG but much lesser to other agonists (Fig. S2). Next, we compared the capabilities of DCs inducing IFN-λs and IFN-β genes in response to relevant TLR agonists. BDCA3$^+$ DCs expressed extremely high levels of IL-29, IL-28A, and IL-28B transcripts compared to other DCs, whereas pDCs induced a higher level of IFN-β than other DCs (Fig. S3A).

Similar results were obtained with the protein levels of IFN-λs, IFN-β, and IFN-α released from DC subsets stimulated with TLR agonists. BDCA3$^+$ DCs produce significantly higher levels of IL-29, IL-28B, and IL-28A than the other DC subsets. In clear contrast, pDCs release a significantly larger amount of IFN-β and IFN-α than BDCA3$^+$ DCs or mDCs (Fig. 3A, Fig. S3B). As for the relationship among the quantity of IFN-λ subtypes from poly IC-stimulated BDCA3$^+$ DCs, the levels of IL-29/IFN-λ1 and IL-28B/IFN-λ3 were positively correlated ($R^2 = 0.76$, $P < 0.05$), and those of IL-28A/IFN-λ2 and IL-28B/IFN-λ3 were positively correlated as well ($R^2 = 0.84$, $P < 0.0005$), respectively (Fig. S3C). These results show that the transcription and translation machineries of IFN-λs may be overlapped among IFN-λ subtypes in BDCA3$^+$ DCs upon poly IC stimulation.

Liver BDCA3$^+$ DCs sorted from IHLs possess the ability to produce IL-28B in response to poly IC (Fig. 3B), showing that they are comparably functional. In response to poly IC, BDCA3$^+$ DCs were capable of producing inflammatory cytokines as well, such as TNF-α, IL-6, and IL-12p70 (Fig. S4A). By using Huh7 cells harboring HCV subgenomic replicons (HCV-N, genotype 1b), we confirmed that the supernatants from poly IC-stimulated BDCA3$^+$ DCs suppressed HCV replication in an IL-28B concentration-dependent manner (Fig. S4B). Therefore, poly IC-stimulated BDCA3$^+$ DCs are capable of producing biologically active substances suppressing HCV replication, some part of which may be mediated by IFN-λs.

BDCA3$^+$ DCs Produce IL-28B upon HCVcc or HCV/JFH-1-Transfected Huh7.5.1 Cells. We stimulated freshly isolated BDCA3$^+$ DCs, pDCs and mDCs with infectious viruses, such as HCVcc, Japanese encephalitis virus (JEV), and herpes simplex virus (HSV). In preliminary experiments, we confirmed that HCVcc stimulated BDCA3$^+$ DCs to release IL-28B in a dose-dependent manner (Fig. S5). BDCA3$^+$ DCs

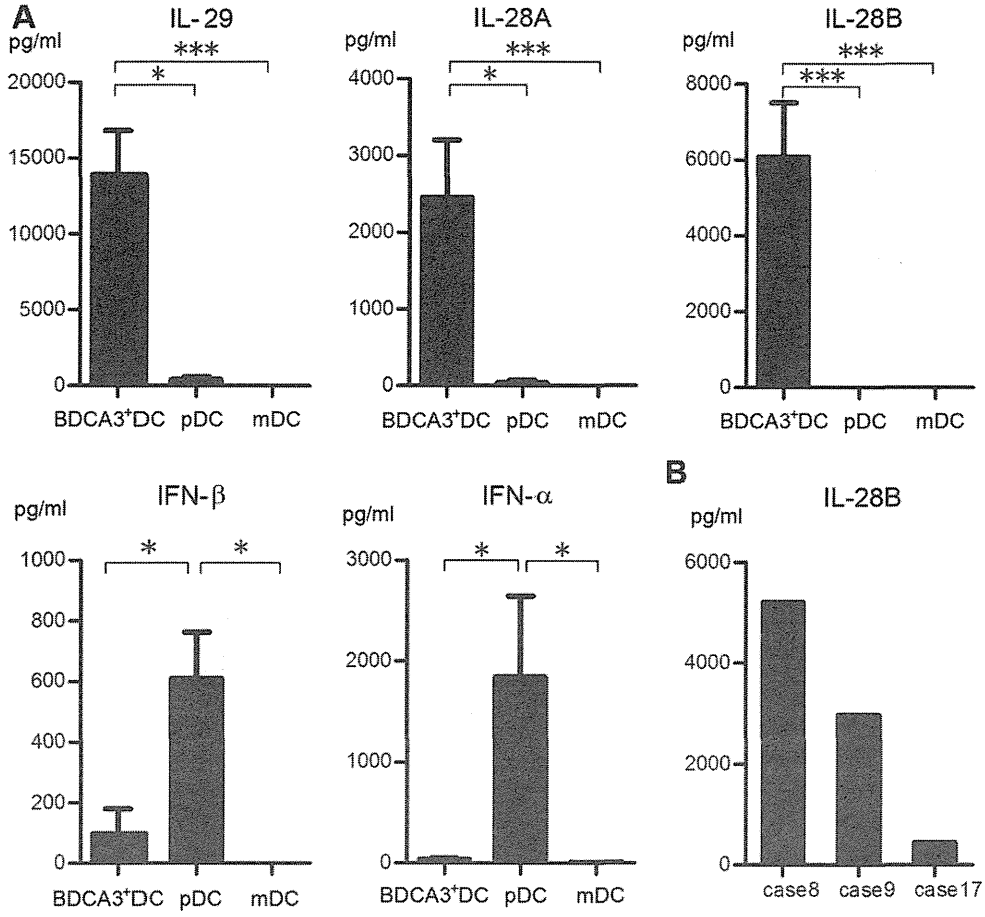


Fig. 3. BDCA3$^+$ DCs recovered from peripheral blood or intrahepatic lymphocytes produce large amounts of IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B/IFN-λ3 in response to poly IC. (A) BDCA3$^+$ DCs and mDCs were cultured at 2.5 $\times$ 10^4 cells with 25 mg/mL poly IC, and pDCs were with 5 mM CPG for 24 hours. The supernatants were examined for IL-29, IL-28A, IL-28B, IFN-β and IFN-α. Results are shown as mean $\pm$ SEM from 15 experiments. *P < 0.05; ***P < 0.0005 by Kruskal-Wallis test. (B) For the IL-28B production, BDCA3$^+$ DCs in intrahepatic lymphocytes were cultured at 2.5 $\times$ 10^4 cells with 25 mg/mL poly IC for 24 hours. The samples of cases 8 and 9 were obtained from patients with non-B, non-C liver disease and that of case 17 was from an HCV-infected patient (Supporting Table 1).

produced a large amount of IL-28B upon exposure to HCVcc and released a lower amount of IFN-α upon HCVcc or HSV (Fig. 4A). In contrast, pDCs produced a large amount of IFN-α in response to HCVcc and HSV and a much lower level of IL-28B upon HCVcc (Fig. S6). In mDCs, IL-28B and IFN-α were not detectable with any of these viruses (data not shown).

BDCA3$^+$ DCs produced significantly higher levels of IL-28B than the other DCs upon HCVcc stimulation (Fig. 4B). By contrast, HCVcc-stimulated pDCs released significantly larger amounts of IFN-β and IFN-α than the other subsets (Fig. 4B). Liver BDCA3$^+$ DCs were capable of producing IL-28B in response to HCVcc (Fig. 4C). These results show that, upon HCVcc stimulation, BDCA3$^+$ DCs produce more IFN-λs and pDCs release more IFN-β and IFN-α than the other DC subsets, respectively. Taking a clinical impact of IL-28B genotypes on HCV eradication into consideration, we focused on IL-28B/IFN-λ3 as a representative for IFN-λs in the following experiments.

In a coculture with JFH-1-infected Huh7.5.1 cells, BDCA3$^+$ DCs profoundly released IL-29, IL-28A, and IL-28B (Fig. 4D, the results of IL-29 and IL-28A, not shown), whereas BDCA3$^+$ DCs failed to respond to Huh7.5.1 cells lacking HCV/JFH-1, showing that IL-28B production from BDCA3$^+$ DCs is dependent on HCV genome (Fig. 4D). In the absence of BDCA3$^+$ DCs, IL-28B is undetectable in the supernatant from JFH-1-infected Huh7.5.1 cells, demonstrating that BDCA3$^+$ DCs, not HCV-replicating Huh7.5.1 cells, produce detectable amount of IL-28B (Fig. 4D). In the coculture, BDCA3$^+$ DCs comparably released IL-28B either in the presence or the absence of transwells, suggesting that cell-to-cell contact between DCs and Huh7.5.1 cells is dispensable for IL-28B response (Fig. 4E). In parallel with the quantity of IL-28B in the coculture, ISG15 was significantly induced only in JFH-1-infected Huh7.5.1 cells cocultured with BDCA3$^+$ DCs (Fig. 4F). A strong induction was observed with other ISGs in JFH-1-infected Huh7.5.1 in the presence of BDCA3$^+$ DCs, such as IFIT1, MxA, RSD2, IP-10, and USP18 (Fig. S7). The results clearly show that BDCA3$^+$ DCs are capable of producing large amounts of IFN-λs in response to cellular or cell-free HCV, thereby inducing various ISGs in bystander liver cells.

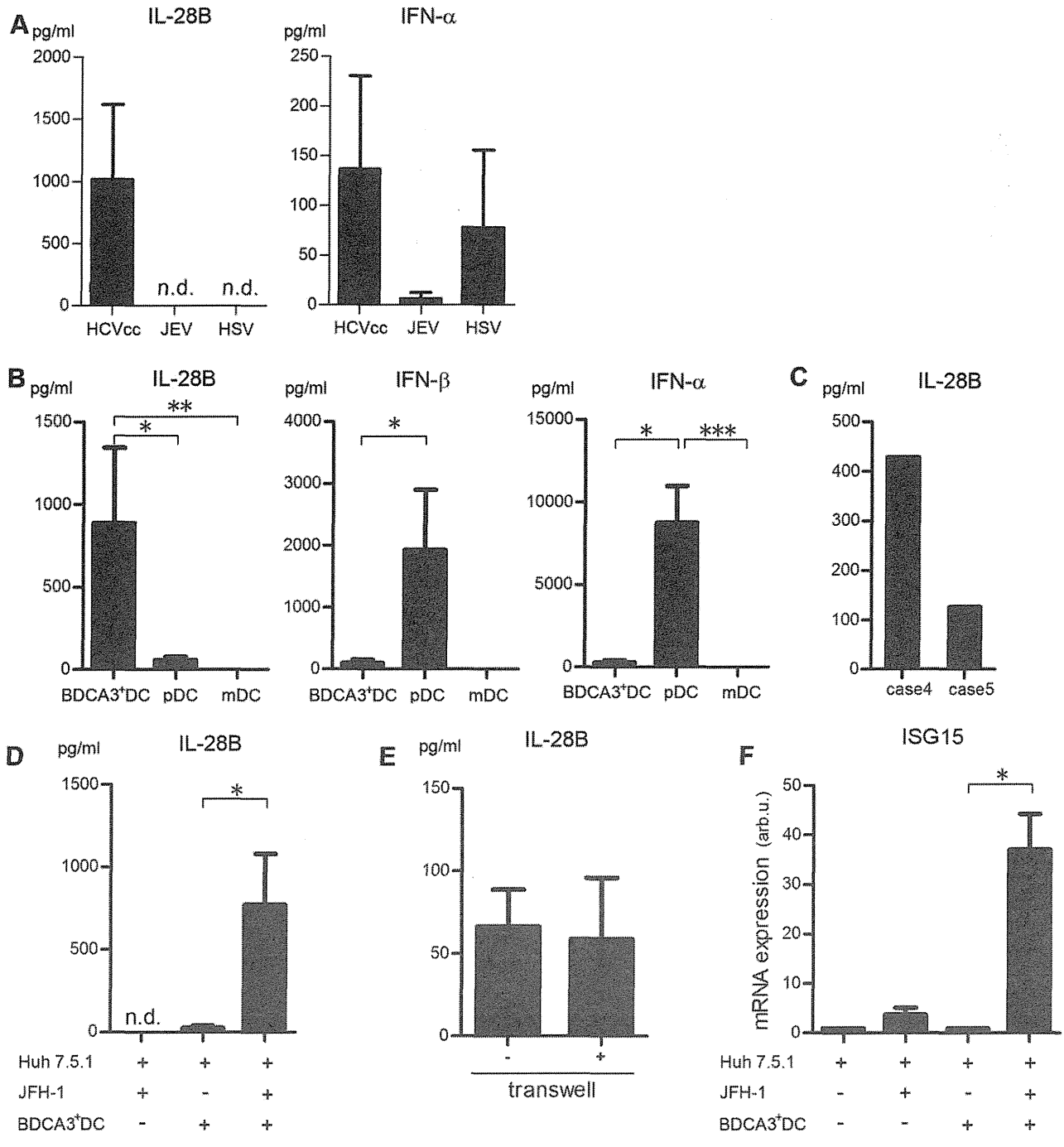


Fig. 4. BDCA3$^+$ DCs produce IL-29, IL-28A, and IL-28B upon cell-cultured HCV or HCV/JFH-1-transfected Huh7.5.1 cells, thereby inducing ISG. (A) BDCA3$^+$ DCs were cultured at 2.5 $\times$ 10^4 cells for 24 hours with HCVcc, JEV, or HSV at a multiplicity of infection (MOI) of 10. Results are shown as mean $\pm$ SEM from six experiments. n.d.; not detected. (B) BDCA3$^+$ DCs, pDCs, and mDCs were cultured at 2.5 $\times$ 10^4 cells for 24 hours with HCVcc at an MOI of 10. The results are shown as mean $\pm$ SEM from 11 experiments. *$P < 0.05$; **$P < 0.0005$; ***$P < 0.0005$ by Kruskal-Wallis test. (C) BDCA3$^+$ DCs recovered from intrahepatic lymphocytes were cultured at 2.5 $\times$ 10^4 cells for 24 hours with HCVcc at an MOI of 10. Both of the samples (cases 4 and 5) were obtained from patients with non-B, non-C liver disease. (D,E) BDCA3$^+$ DCs were cocultured at 2.5 $\times$ 10^4 cells with JFH-1-transfected (MOI = 2) or -untransfected Huh7.5.1 cells for 24 hours. The supernatants of JFH-1-transfected Huh7.5.1.cells without BDCA3$^+$ DCs were also examined. In some experiments of the coculture with JFH-1-transfected Huh7.5.1 cells and BDCA3$^+$ DCs, transwells were inserted into the wells (E). Results are shown as mean $\pm$ SEM from five experiments. *$P < 0.05$ by paired t test. (F) BDCA3$^+$ DCs were cocultured at 2.5 $\times$ 10^4 cells with JFH-1-transfected Huh7.5.1 cells (MOI = 2) or -untransfected Huh7.5.1 cells for 24 hours. The Huh7.5.1.cells were harvested and subjected to real-time RT-PCR analyses for ISG15 expression. The results are shown as mean $\pm$ SEM from five experiments. *$P < 0.05$ by paired t test. HCVcc, cell-cultured HCV; JEV, Japanese encephalitis virus; HSV, herpes simplex virus.

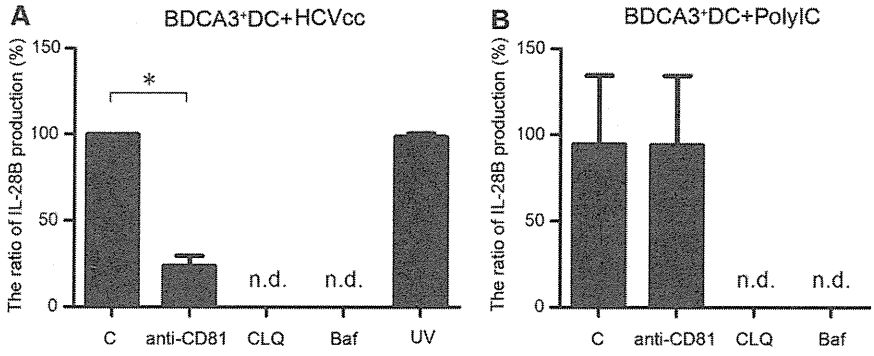


Fig. 5. The CD81 and endosome acidification is involved in the production of IL-28B from HCV-stimulated BDCA3$^+$ DCs, but HCV replication is not necessary. (A,B) BDCA3$^+$ DCs were cultured at 2.5 $\times$ 10^4 cells with HCVcc at an MOI of 10 (A) or poly IC (25 μg/mL) (B). In some experiments, UV-irradiated HCVcc was used at the same MOI, and BDCA3$^+$ DCs were treated with anti-CD81Ab (5 mg/mL), chloroquine (10 mM), or bafilomycin A1 (25 nM). The results are expressed as ratios of IL-28B quantity with or without the treatments. They are shown as mean $\pm$ SEM from five experiments. *$P < 0.05$ by paired t test. C, control; CLQ, treatment with chloroquine; Baf, treatment with bafilomycin A1; UV, ultraviolet-irradiated HCVcc; n.d., not detected.

CD81 and Endosome Acidification Are Involved in IL-28B Production from HCV-Stimulated BDCA3$^+$ DCs, but HCV Replication Is Not Involved. It is not known whether HCV entry and subsequent replication in DCs is involved or not in IFN response.[18,19] To test this, BDCA3$^+$ DCs were inoculated with UV-irradiated, replication-defective HCVcc. We confirmed that UV exposure under the current conditions is sufficient to negate HCVcc replication in Huh7.5.1 cells, as demonstrated by the lack of expression of NS5A after inoculation (data not shown). BDCA3$^+$ DCs produced comparable levels of IL-28B with UV-treated HCVcc, indicating that active HCV replication is not necessary for IL-28B production (Fig. 5A).

We next examined whether or not the association of HCVcc with BDCA3$^+$ DCs by CD81 is required for IL-28B production. It has been reported that the E2 region of HCV structural protein is associated with CD81 on cells when HCV enters susceptible cells.[13,20] We confirmed that all DC subsets express CD81, the degree of which was most significant on BDCA3$^+$ DCs (Fig. 1B, Fig. S1). Masking of CD81 with Ab significantly impaired IL-28B production from HCVcc-stimulated BDCA3$^+$ DCs in a dose-dependent manner (Fig. 5A, Fig. S8), suggesting that HCV-E2 and CD81 interaction is involved in the induction. The treatment of poly IC-stimulated BDCA3$^+$ DCs with anti-CD81 Ab failed to suppress IL-28B production (Fig. 5B).

HCV enters the target cells, which is followed by fusion steps within acidic endosome compartments. Chloroquine and bafilomycin A1 are well-known and broadly used inhibitors of endosome TLRs, which are reported to be capable of blocking TLR3 response in human monocyte-derived DC.[21,22] In our study, the treatment of BDCA3$^+$ DCs with chloroquine, bafilomycin A1, or NH4Cl significantly suppressed their IL-28B production either in response to HCVcc or poly IC (Fig. 5A,B, NH4Cl, data not shown). These results suggest that the endosome acidification is involved in HCVcc- or poly IC-stimulated BDCA3$^+$ DCs to produce IL-28B. The similar results were obtained with HCVcc-stimulated pDCs for the production of IL-28B (Fig. S9). We validated that such concentration of chloroquine (10 mM) and bafilomycin A1 (25 nM) did not reduce the viability of BDCA3$^+$ DCs (Fig. S10).

BDCA3$^+$ DCs Produce IL-28B in Response to HCVcc by a TIR-Domain-Containing Adapter-Inducing Interferon-β (TRIF)-Dependent Mechanism. TRIF/TICAM-1, a TIR domain-containing adaptor, is known to be essential for the TLR3-mediated pathway.[23] In order to elucidate whether TLR3-dependent pathway is involved or not in IL-28B response of BDCA3$^+$ DCs, we added the cell-permeable TRIF-specific inhibitory peptide (Invivogen) or the control peptide to poly IC- or HCVcc-stimulated BDCA3$^+$ DCs. Of particular interest, the TRIF-specific inhibitor peptide, but not the control one, significantly suppressed IL-28B production from poly IC- or HCVcc-stimulated BDCA3$^+$ DCs (Fig. 6A,B). In clear contrast, the TRIF-specific inhibitor failed to suppress IL-28B from HCVcc-stimulated pDCs (Fig. 6C), suggesting that pDCs recognize HCVcc in an endosome-dependent but TRIF-independent pathway. These results show that BDCA3$^+$ DCs may recognize HCVcc by way of the TRIF-dependent pathway to produce IL-28B.

BDCA3$^+$ DCs in Subjects with IL-28B Major Genotype Produce More IL-28B in Response to HCV than Those with IL-28B Minor Type. In order to compare the ability of BDCA3$^+$ DCs to release IL-28B in healthy subjects between IL28B major (rs8099917, TT)

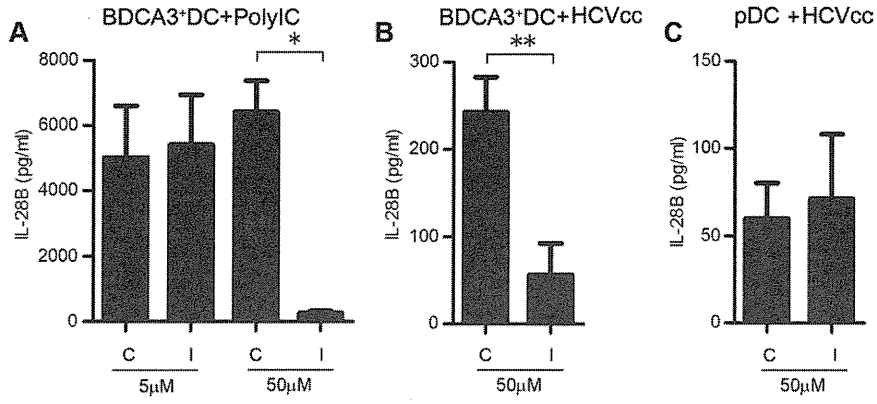


Fig. 6. BDCA3$^+$ DCs produce IL-28B upon HCVcc stimulation in a TRIF-dependent mechanism. BDCA3$^+$ DCs or pDCs had been treated with 5 or 50 mM TRIF inhibitory peptide or control peptide for 2 hours. Subsequently, BDCA3$^+$ DCs were stimulated with Poly IC (25 μg/mL) or HCVcc (MOI = 10), and pDCs were stimulated with HCVcc (MOI = 10), respectively. IL-28B was quantified by ELISA. They are shown as mean ± SEM from five experiments. *$P < 0.05$ by paired t test. C, TRIF control peptide; I, TRIF inhibitory peptide.

and minor hetero (TG) genotypes, we stimulated BDCA3$^+$ DCs of the identical subjects with poly IC (25 mg/mL, 2.5 mg/mL, 0.25 mg/mL), HCVcc or JFH-1-infected Huh 7.5.1, and subjected them to ELISA. The levels of IL-28B production by poly IC-stimulated BDCA3$^+$ DCs were comparable between subjects with IL-28B major and minor type (Fig. 7A). Similar results were obtained with the lesser concentrations of poly IC (Fig. S11). Of particular interest, in response to HCVcc or JFH-1 Huh7.5.1 cells, the levels of IL-28B from BDCA3$^+$ DCs were significantly higher in subjects with IL-28B major than those with minor type (Fig. 7B,C, S12).

Discussion

In this study we demonstrated that human BDCA3$^+$ DCs (1) are present at an extremely low frequency in PBMC but are accumulated in the liver; (2) are capable of producing IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B/IFN-λ3 robustly in response to HCV; (3) recognize HCV by a CD81-, endosome acidification and TRIF-dependent mechanism; and (4) produce larger amounts of IFN-λs upon HCV stimulation in subjects with IL-28B major genotype (rs8099917, TT). These characteristics of BDCA3$^+$ DCs are quite unique in comparison with other DC repertoires in the settings of HCV infection.

At the steady state, the frequency of DCs in the periphery is relatively lower than that of the other immune cells. However, under disease conditions or physiological stress, activated DCs dynamically migrate to the site where they are required to be functional. However, it remains obscure whether functional BDCA3$^+$ DCs exist or not in the liver. We identified BDCA3$^+$CLEC9A$^+$ cells in the liver tissue (Fig. 1D). In a paired frequency analysis of BDCA3$^+$ DCs between in PBMCs and in IHLs, the cells are more abundant in the liver. The phenotypes of liver BDCA3$^+$ DCs were more mature than the PBMC counterparts. In support of our observations, a recent publication showed that CD141$^+$ (BDCA3$^+$) DCs are accumulated and more mature in the liver, the trend of which is more in HCV-infected liver.[24] We confirmed that liver BDCA3$^+$ DCs are functional, capable of releasing IFN-λs in response to poly IC or HCVcc.

BDCA3$^+$ DCs were able to produce large amounts of IFN-λs but much less IFN-β or IFN-α upon TLR3 stimulation. In contrast, in response to TLR9 agonist,

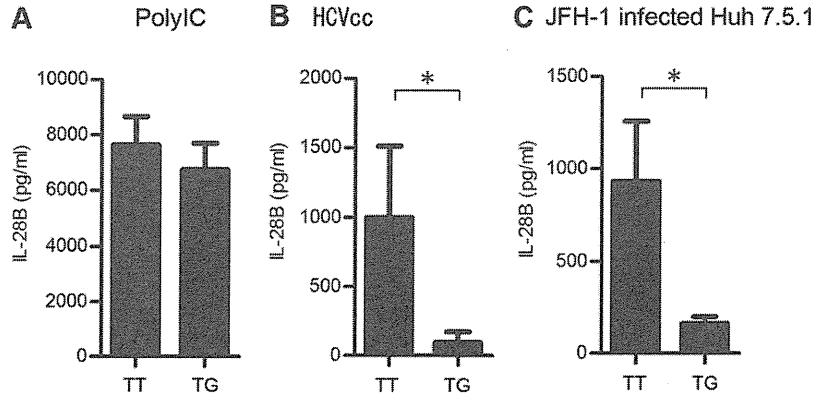


Fig. 7. In response to HCVcc, BDCA3$^+$ DCs of healthy donors with IL-28B major genotype (rs8099917, TT) produced more IL-28B than those with minor type (TG). BDCA3$^+$ DCs of healthy donors with IL-28B TT (rs8099917) or TG genotype were cultured at 2.5 × 10^4 cells with 25 mg/mL poly IC (A), with HCVcc at an MOI of 10 (B), or with JFH-1-infected Huh 7.5.1 cells (C) for 24 hours. The supernatants were subjected to IL-28B ELISA. The same healthy donors were examined for distinct stimuli. The results are the mean ± SEM from 15 donors with TT and 8 with TG, respectively. *$P < 0.05$ by Mann-Whitney U test.

pDCs released large amounts of IFN-β and IFN-α but much less IFN-λs. Such distinctive patterns of IFN response between BDCA3$^+$ DCs and pDCs are of particular interest. It has been reported that interferon regulatory factor (IRF)-3, IRF-7, or nuclear factor kappa B (NF-κB) are involved in IFN-β and IFN-λ1, while IRF-7 and NF-κB are involved in IFN-α and IFN-λ2/λ3.[5] Presumably, the stimuli with TLR3/retinoic acid-inducible gene-I (RIG-I) (poly IC) or TLR9 agonist (CpG-DNA) in DCs are destined to activate these transcription factors, resulting in the induction of both types of IFN at comparable levels. However, the results of the present study did not agree with such overlapping transcription factors for IFN-λs, IFN-β, and IFN-α. Two possible explanations exist for different levels of IFN-λs and IFN-α production by BDCA3$^+$ DCs and pDCs. First, the transcription factors required for full activation of IFN genes may differ according to the difference of DC subsets. The second possibility is that since type III IFN genes have multiple exons, they are potentially regulated by post-transcriptional mechanisms. Thus, it is possible that such genetic and/or posttranscriptional regulation is distinctively executed between BDCA3$^+$ DCs and pDCs. Comprehensive analysis of gene profiles downstream of TLRs or RIG-I in BDCA3$^+$ DCs should offer some information on this important issue.

BDCA3$^+$ DCs were found to be more sensitive to HCVcc than JEV or HSV in IL-28B/IFN-λ3 production. Such different strengths of IL-28B in BDCA3$^+$ DCs depending on the virus suggest that different receptors are involved in virus recognition. Again, the question arises of why BDCA3$^+$ DCs produce large amounts of IFN-λs compared to the amounts produced by pDCs in response to HCVcc. Considering that IRF-7 and NF-κB are involved in the transcription of the IL-28B gene, it is possible that BDCA3$^+$ DCs successfully activate both transcription factors upon HCVcc for maximizing IL-28B, whereas pDCs fail to do so. In support for this possibility, in pDCs it is reported that NF-κB is not properly activated upon HCVcc or hepatoma cell-derived HCV stimulations.[25]

In the present study we demonstrated that HCV entry into BDCA3$^+$ DCs through CD81 and subsequent endosome acidification are critically involved in IL-28B responses. Involvement of TRIF-dependent pathways in IL-28B production was shown by the significant inhibition of IL-28B with TRIF inhibitor. Nevertheless, active HCV replication in the cells is not required. Based on our data, we considered that BDCA3$^+$ DCs recognize HCV genome mainly by an endosome and TRIF-dependent mechanism. Although the results with UV-irradiated HCVcc, anti-CD81 blocking Ab, and chloroquine were quite similar, the TRIF-specific inhibitor failed to suppress IL-28B from pDCs (Fig. 6, Fig. S9).

In the coculture with JFH-transfected Huh7.5.1 cells, BDCA3$^+$ DCs presumably receive some signals for IL-28B production by way of cell-to-cell dependent and independent mechanisms. In the present study, most of the stimuli to BDCA3$^+$ DCs for IL-28B production may be the released HCVcc from Huh7.5.1 cells, judging from the inability of suppression with transwells. However, a contribution of contact-dependent mechanisms cannot be excluded in the coculture experiments. HCV genome is transmissible from infected hepatocytes to uninfected ones through tight junction molecules, such as claudin-1 and occuludin. Further investigation is needed to clarify whether such cell-to-cell transmission of viral genome is operated or not in BDCA3$^+$ DCs.

The relationship between IL-28B expression and the induction of ISGs has been drawing much research attention. In primary human hepatocytes, it is reported that HCV primarily induces IFN-λ, instead of type-I IFNs, subsequently enhancing ISG expression.[7] Of particular interest is that the level of hepatic IFN-λs is closely correlated with the strength of ISG response.[26] These reports strongly suggest that hepatic IFN-λs are a crucial driver of ISG induction and subsequent HCV eradication. Besides, it is likely that BDCA3$^+$ DCs, as a bystander IFN-λ producer in the liver, have a significant impact on hepatic ISG induction. In support of this possibility, we demonstrated in this study that BDCA3$^+$ DCs are capable of producing large amounts of IFN-λs in response to HCV, thereby inducing ISGs in the coexisting liver cells.

Controversial results have been reported regarding the relationship between IL28B genotypes and the levels of IL-28 expression. Nevertheless, in chronic hepatitis C patients with IL-28B major genotype, the IL-28 transcripts in PBMCs are reported to be higher than those with minor genotype.[2] In this study, by focusing on a prominent IFN-λ producer (BDCA3$^+$ DCs) and using the assay specific for IL-28B, we showed that the subjects with IL-28B major genotype could respond to HCV by releasing more IL-28B. Of interest, such a superior capacity of BDCA3$^+$ DCs was observed only in response to HCV but not to poly IC. Since the pathways downstream of TLR3-TRIF leading to IL-28B in BDCA3$^+$ DCs should be the same, either HCV or poly IC stimulation, two plausible explanations exist for such a distinct IL-28B response. First, it is possible that distinct epigenetic regulation may be

involved in IL-28B gene according to the IL-28B genotypes. Recently, in influenza virus infection, it is reported that micro-RNA29 and DNA methyltransferase are involved in the cyclooxygenase-2-mediated enhancement of IL-29/IFN-λ1 production.[27] This report supports the possibility that similar epigenetic machineries could be operated as well in HCV-induced IFN-λs production. Second, it is plausible that the efficiency of the stimulation of TLR3-TRIF may be different between the IL-28B genotypes. Since HCV reaches endosome in BDCA3$^+$ DCs by way of the CD81-mediated entry and subsequent endocytosis pathways, the efficiencies of HCV handling and enzyme reactions in endosome may be influential in the subsequent TLR3-TRIF-dependent responses. Certain unknown factors regulating such process may be linked to the IL-28B genotypes. For a comprehensive understanding of the biological importance of IL-28B in HCV infection, such confounding factors, if they exist, need to be explored.

In conclusion, human BDCA3$^+$ DCs, having a tendency to accumulate in the liver, recognize HCV and produce large amounts of IFN-λs. An enhanced IL-28B/IFN-λ3 response of BDCA3$^+$ DCs to HCV in subjects with IL-28B major genotype suggests that BDCA3$^+$ DCs are one of the key players in anti-HCV innate immunity. An exploration of the molecular mechanisms of potent and specialized capacity of BDCA3$^+$ DCs as IFN-λ producer could provide useful information on the development of a natural adjuvant against HCV infection.

References

1. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-1104.
2. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.
3. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
4. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798-801.
5. Kotenko SV. IFN-lambdas. Curr Opin Immunol 2011;23:583-590.
6. Urban TJ, Thompson AJ, Bradrick SS, Fellay J, Schuppan D, Cronin KD, et al. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. Hepatology 2010;52:1888-1896.
7. Park H, Serti E, Eke O, Muchmore B, Prokunina-Olsson L, Capone S, et al. IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. Hepatology 2012;56:2060-2070.
8. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature 2007;449:819-826.
9. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 2001;106:259-262.
10. Poulin LF, Salio M, Griessinger E, Anjos-Afonso F, Craciun L, Chen JL, et al. Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8alpha+ dendritic cells. J Exp Med 2010;207:1261-1271.
11. Lauterbach H, Bathke B, Gilles S, Traidl-Hoffmann C, Luber CA, Fejer G, et al. Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. J Exp Med 2010;207:2703-2717.
12. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
13. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
14. Mori Y, Okabayashi T, Yamashita T, Zhao Z, Wakita T, Yasui K, et al. Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. J Virol 2005;79:3448-3458.
15. Sugiyama M, Kimura T, Naito S, Mukaide M, Shinauchi T, Ueno M, et al. Development of interferon lambda 3 specific quantification assay for its mRNA and serum/plasma specimens. Hepatol Res 2012;42:1089-1099.
16. Schreibelt G, Klinkenberg LJ, Cruz LJ, Tacken PJ, Tel J, Kreutz M, et al. The C type lectin receptor CLEC9A mediates antigen uptake and (cross-)presentation by human blood BDCA3+ myeloid dendritic cells. Blood 2012;119:2284-2292.
17. Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, et al. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med 2010;207:1247-1260.
18. Marukian S, Jones CT, Andrus L, Evans MJ, Ritola KD, Charles ED, et al. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. Hepatology 2008;48:1843-1850.
19. Liang H, Russell RS, Yonkers NL, McDonald D, Rodriguez B, Harding CV, et al. Differential effects of hepatitis C virus JFH1 on human myeloid and plasmacytoid dendritic cells. J Virol 2009;83:5693-5707.
20. Zhang J, Randall G, Higginbottom A, Monk P, Rice CM, McKeating JA. CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. J Virol 2004;78:1448-1455.
21. Blanchard E, Belouzard S, Goueslain L, Wakita T, Dubuisson J, Wychowski C, et al. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J Virol 2006;80:6964-6972.
22. de Bouteiller O, Merck E, Hasan UA, Hubac S, Benguigui B, Trinchieri G, et al. Recognition of double-stranded RNA by human toll-like receptor 3 and downstream receptor signaling requires multimerization and an acidic pH. J Biol Chem 2005;280:38133-38145.
23. Takeda K, Akira S. TLR signaling pathways. Semin Immunol 2004;16:3-9.
24. Velazquez VM, Hon H, Ibegbu C, Knechtle SJ, Kirk AD, Grakoui A. Hepatic enrichment and activation of myeloid dendritic cells during chronic hepatitis C virus infection. Hepatology 2012;56:2071-2081.
25. Dental C, Florentin J, Aouar B, Gondois-Rey F, Durantel D, Baumert TF, et al. Hepatitis C virus fails to activate NF-kappaB signaling in plasmacytoid dendritic cells. J Virol 2012;86:1090-1096.
26. Thomas E, Gonzalez VD, Li Q, Modi AA, Chen W, Noureddin M, et al. HCV infection induces a unique hepatic innate immune response associated with robust production of type III interferons. Gastroenterology 2012;142:978-988.
27. Fang J, Hao Q, Liu L, Li Y, Wu J, Huo X, Zhu Y. Epigenetic changes mediated by microRNA miR29 activate cyclooxygenase 2 and lambda-1 interferon production during viral infection. J Virol 2012;86:1010-1020.

＜総　説＞

B型肝炎ウイルス感染における宿主免疫応答の重要性 —特にNKT細胞の関与について—

村田　一素*

索引用語：　B型急性肝炎　　自然免疫　　獲得免疫　　NKT細胞　　脂質抗原

はじめに

B型肝炎ウイルス（HBV）感染者は，全世界で約2億人いると推定されている．そのうち年間約60万人の患者がHBV関連で死亡しており，HBVは世界における重要感染症の1つである[1]．HBVは，1965年にBlumbergらにより肝炎の原因ウイルスとして発見されて以来[2]，約半世紀にわたり多くの基礎的・臨床的研究が進められてきた．HBVワクチンによる母子感染予防の成功[3]や，近年の核酸アナログの普及により肝障害のコントロール・肝癌発症抑制がある程度可能となってきたことなどによりHBV感染者の予後が改善しつつある[4]．しかし，今なおHBVによる肝炎発症機構，肝癌発症機構などの詳細は不明であり，かつ現段階ではウイルス排除に至る治療法はない．

B型急性肝炎の病態と経過はウイルス側因子[5)~9)]と宿主側因子で規定されている．思春期以降にHBVに感染し急性肝炎もしくは劇症肝炎を発症した場合，大多数は一過性感染で終了し，慢性肝炎に移行することは少ない．HBV自体は肝障害を起こさない非細胞障害性ウイルス（non-cytopathic virus）であることから[10)11)]，HBVにおける肝障害機序としてHBVに対する宿主の免疫応答が重要視されている．HBVは非細胞障害性ウイルスであるが故に，HBV持続感染時でも無症候性キャリアが存在し，またHBVの急性感染潜伏期では，ほとんどの肝細胞が感染しているにも関わらず生化学的・組織学的に異常を認めない[12]．また，HBVの持続感染が成立するのは，母子感染のように免疫が確立していない幼小児や免疫力が低下している高齢者であること，悪性リンパ腫をはじめとする悪性新生物やリウマチ疾患に対して免疫を強力に抑制するような生物学的製剤を投与した場合のHBVの再活性化など[13)~17)]，HBVと免疫は強く関与している．最近ではgenome-wide association study（GWAS）の解析より，B型肝炎の慢性化因子として宿主免疫応答に関与するhuman leukocyte antigen（HLA）-DPが同定されている[18)19)]．

一般に生体は2種類の免疫応答によって微生物の侵入を防御している．自然免疫（innate immunity）は主にtoll-like receptors（TLR）[20)]やretinoic acid inducible gene-I（RIG-I）[21)]のようなpattern recognition receptorsを介して微生物を認識し，数分でマクロファージやnatural killer（NK）細胞を活性化する[22)]．一方，獲得免疫（adaptive immunity）は，抗原特異的T細胞あるいはB細胞が感染細胞を攻撃し，アポトーシスの誘導を行う．しかし，その効果が発揮されるためには，それぞれの細胞がクローン性に増殖する必要があるため，少なくとも数日を要する．HBV制御において獲得免疫は重要であるが，効率的な獲得免疫の起動にはHBV感染早期の自然免疫応答が非常に大きな役割を果たしている[10)23)]．

近年，早期自然免疫応答の責任細胞としてnatural killer T（NKT）細胞が注目されている．そこで，我々はB型急性肝炎時のNKT細胞の活性化機序に関して，培養細胞およびマウスを用いて検討した．その結果，急性HBV感染により肝細胞に誘導された脂質抗原（lipid antigen）が，NKT細胞に対して抗原提示を行うCD1d分子上に提示されることによってNKT細胞が活性化し，さらに活性化したNKT細胞がNK細胞，T細胞，B細胞を活性化することにより，HBV感染を鎮静化させることを発見し報告した[24)]．

本稿ではHBVにおける免疫学的研究の歴史を辿るとともに，我々の知見を含めた現時点におけるHBVに対する宿主免疫応答の見解について概説する．

国際医療研究センター国府台病院肝炎・免疫研究センター
*Corresponding author: dmurata@hospk.ncgm.go.jp
＜受付日2012年8月29日＞＜採択日2012年11月13日＞

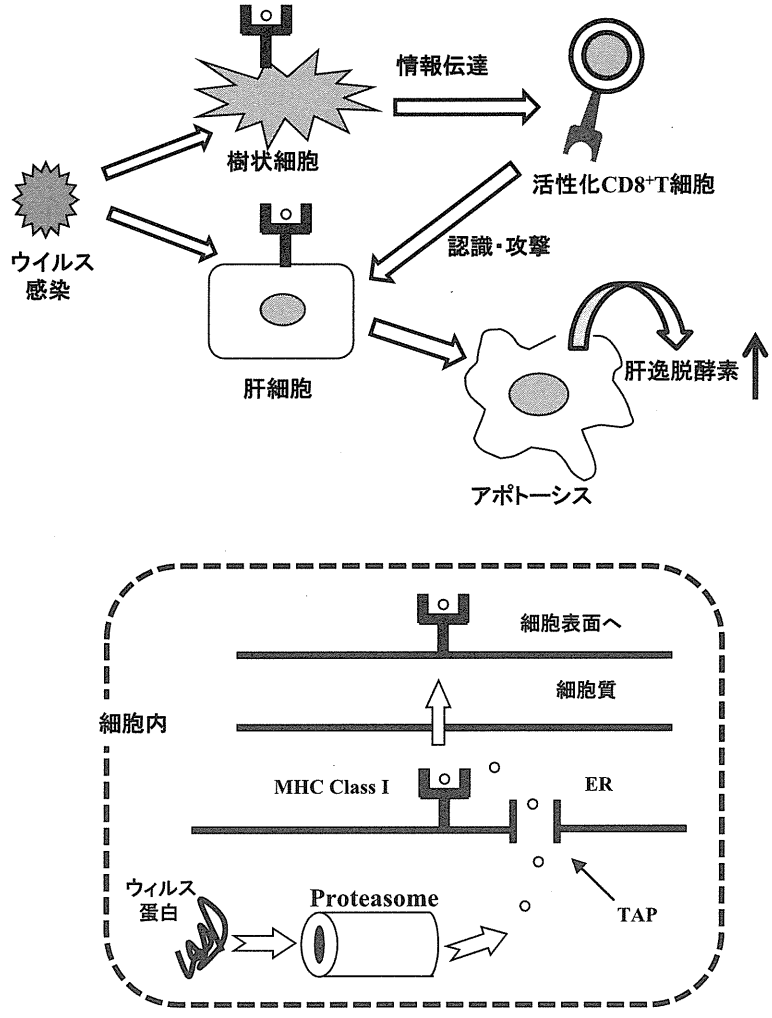


Fig. 1　細胞障害性T細胞の活性化機序
ER：endoplasmic reticulum
TAP：transporters associated with antigen processing

1. HBV感染時の獲得免疫応答（adaptive immunity）

獲得免疫には，主にCD4⁺T細胞（ヘルパーT細胞），CD8⁺T細胞（キラーT細胞）が関与している．免疫監視細胞である樹状細胞や標的細胞（HBVでは肝細胞）にウイルスが感染すると細胞内endoplasmic reticulum（ER）の蛋白合成酵素複合体であるproteasomeにて数個のアミノ酸（ペプチド）に断片化される[25)26)]．断片化された抗原ペプチドは粗面小胞体の膜に存在するTAP（transporter associated with antigen processing）と呼ばれるレセプターを介してMHC class I上に提示されると抗原ペプチド・MHC class I複合体は細胞表面に移動する[27)28)]．CD8⁺T細胞はT cell receptorを用いて抗原ペプチド・MHC class I複合体を認識し活性化されると，同じ抗原ペプチドがMHC class I上に提示されている感染細胞を認識し，アポトーシスに導き，感染細胞もろともウイルスを排除する（Fig. 1）．一方，マクロファージなどに貪食されたウイルス蛋白はMHC class II上にペプチドとして抗原提示され，MHC class II分子とHBV由来ペプチドの複合体がCD4⁺T細胞に認識される[29)]．CD4⁺T細胞はサイトカイン産生により直接ウイルス制御を行うとともに，抗原特異的CD8⁺T細胞の誘導を補

助し，かつB細胞による抗体産生に関わる．このように宿主の免疫応答は，effector細胞が法則性を持って抗原・抗原提示細胞複合体を認識し，活性化されることにより起こる．

獲得免疫に関して，HBVワクチン接種者[30]やB型慢性肝炎患者[31]の末梢血中にHBV特異的T細胞が出現することが報告されていたが，そのことと肝障害との関わりについての直接的な証拠はなかった．Moriyamaらは，HBsAg遺伝子導入マウス（HBsAg transgenic（Tg）マウス）に，同種同系マウスにHBsAg遺伝子導入ワクシニアウイルス（vvHBsAg）を感染させて得られたHBsAg特異的細胞障害性T細胞(cytotoxic T lymphocytes；CTL）を移入した場合に急性肝障害が生じることを報告した[32]．このことは，MHC class I上にHBsAg抗原関連ペプチドが提示されたHBsAg Tgマウスの肝細胞（標的細胞）をHBsAg特異的CTLが認識し，同細胞をアポトーシスに誘導したと考えられる．この論文はHBVにおける肝障害機序としてCTLの重要性を直接的に証明したものとして重要である．さらにAndoらはHBsAg特異的CTLクローンをHBsAg Tgマウスに移入し，継時的に観察することにより，CTLがHBsAg抗原ペプチド提示肝細胞を認識して肝細胞にアポトーシスを誘導するだけでなく，抗原非特異的なリンパ球や白血球などの炎症細胞がCTLの効果を増幅し，さらにCTLより放出されたinterferon（IFN)-γやtumor necrosis factor(TNF)-αが局所のマクロファージを活性化し，周辺肝細胞を破壊することを報告した[33]．HBV特異的CTLの全リンパ球に占める頻度は少ないにも関わらず，広範囲な肝障害が誘導される機序を示したことで重要な意味を持つが，最後のステップは肝細胞にHBsAg(large S)の蓄積のみられる系統(HBsAg Tgマウス)のみに観察されると考えられている．興味深いことにパーフォリンノックアウトマウス（CTLの細胞障害に必要なパーフォリンは欠如しているもののIFN-γとTNF-α産生能は保たれている)にて誘導したHBsAg特異的CTLをHBV全遺伝子を含み肝臓内でのHBV複製が起こるHBV遺伝子導入マウス(HBV Tg）に移入した場合，肝障害を来さずに抗HBV効果を示すが，同マウスにIFN-γ抗体とTNF-α抗体を同時に投与した後に野生型マウスからのHBsAg特異的CTLを移入すると肝障害は来すものの抗HBV効果が消失した[34]．これらのことから，この実験における抗HBV効果はHBsAg特異的CTLによる標的細胞の破壊（アポトーシス)によるよりも，CTLから分泌されたIFN-γ, TNF-αによって媒介される非細胞障害性の効果と考えられている[34]．

HBV感染患者における獲得免疫に関しては，多くの研究が精力的になされている．B型急性肝炎自然治癒症例あるいは慢性肝炎治療成功例においては，MHC class I[35)~39)]およびclass II[40)41)]拘束性T細胞反応が強く起こっている．一方，HBV持続感染時にはHBV特異的T細胞の反応性が低下している[36)~39)42)43)]．また，獲得免疫の活性化により産生されたIFN-α/βもHBV制御に寄与していると考えられている[44)~46)]．これらのことは，HBV感染において，MHC class I拘束性$CD8^+$T細胞，またはMHC class II拘束性$CD4^+$T細胞など宿主免疫応答の反応性の低下が慢性化に関与していることを示している．

2. 急性HBV感染における自然免疫応答（innate immunity）

チンパンジーにHBVを感染させると，HBV DNAは感染後4～7週間で血清および肝組織中に検出される．引き続いてHBVの複製が指数関数的に起こり，血中HBV DNAは感染後8週間でピークに達し，その後急速に低下する．HBV特異的CTLの出現時期を検討すると感染後10週頃より出現，そして16～18週でピークに達し，それに一致して肝逸脱酵素の上昇を認める（Fig. 2)．すなわち，感染10週後より細胞障害性T細胞が誘導され，CTLが感染肝細胞をアポトーシスに誘導し破壊したため，肝逸脱酵素が上昇したと考えられる．ところが，CTLの出現時期より以前にHBV DNA量が低下しているので，ウイルス量を減少させたのはHBV特異的CTLではなく，他の要因があると推測される[47]．この現象は，HBV DNA量は減少するものの肝逸脱酵素の上昇がわずかであることから“細胞障害のないウイルス排除”(non-cytopathic viral clearance）と呼ばれたが[47]，チンパンジーにHBVを投与した2週間までは明らかな免疫遺伝子の誘導がないため，当初はHBVが自然免疫を回避しているためと考えられていた[48]．しかし，その後B型急性肝炎患者リンパ球の解析により，感染わずか数週間においてHBV特異的TおよびB細胞の活性化が確認されたことから[49)50)]，自然免疫システムがHBVを感知し宿主免疫反応を誘導していることが判明した．さらに，ヒトにHBVが，ウッドチャックにウッドチャック肝炎ウイルスが感染した場合，感染早期にNKT細胞が活性化されていることが示され[50)51)]，自然免疫機構の1つとしてNKT細胞が注目されるよう

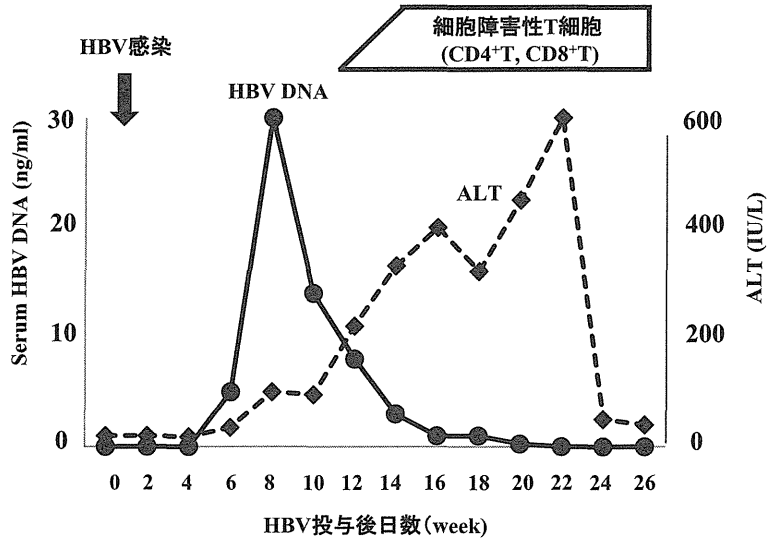


Fig. 2　Non-cytopathic killing
チンパンジーにHBVを感染させると細胞障害性T細胞の発現以前にウイルスは低下している．その際のALT上昇は，わずかである．（文献47より引用改変）

になった．

NK細胞の活性化の増強，NK細胞が産生するIFN-γやTNF-αが，HBV感染早期のHBV増殖抑制に関わっており[34)52)53)]，活性化されたNK細胞は，$CD4^+$T細胞，$CD8^+$T細胞，樹状細胞，NKT細胞など他のリンパ球を活性化させると報告されている[54)]．臨床においてもB型急性肝炎の急性期においてNK細胞数の増加[49)50)]が報告されており，NK細胞も感染早期のHBVコントロールに大きく寄与しているものと考えられる．

TLRは侵入外来抗原であるHBVを認識すると，I型IFNを誘導し，抗HBV効果を示す[55)～57)]．HBV TgにTLR3，TLR4，TLR5，TLR7，TLR9静脈投与すると24時間以内にIFN-α/β依存性にHBVの複製が抑制されると報告されており，B型慢性肝炎の新規治療法としても注目されている．

3. NKT細胞

NKT細胞は通常のT細胞とは異なるT細胞受容体（T cell receptor；TCR）とNK細胞の両マーカーを持ち，MHC class I様のCD1d分子上に提示された脂質抗原を認識する[58)59)]．NKT細胞は肝組織特異的に多く存在し[60)～62)]，マウスでは肝内T細胞の30-50%を占め，脾（3%），末梢血（4%）では少ない．それに比較して，ヒトNKT細胞は，肝内T細胞の0.5%，末梢血T細胞の0.02%と極端に少ない[63)]．

NKT細胞への抗原提示分子であるCD1dは，ヒトとマウスに存在する．しかし，他のCD1ファミリーに属するCD1a，CD1b，CD1cは，ヒトには存在するがマウスでは欠損している[64)]．興味深いことに，CD1d分子はマウスとヒトで90%以上の相同性を認めており[65)]，ヒトNKT細胞はマウスCD1dを認識することができる[66)]．このようにCD1d分子が哺乳類において種を超えて保存されていることは，生体が生存していく上において非常に重要な役割を担っていることを示唆している．CD1dのheavy chainは，MHC class I分子と同様に抗原結合部はα1，α2ドメインで構成されている（Fig. 3）．構造上は類似しているが，MHC class Iがペプチドの結合に適した親水性の溝構造を持つのに対し，CD1d分子は疎水性の溝構造を持ち，糖脂質のアルキル鎖が結合しやすい構造になっている[67)～69)]．また，CD1d分子は肝細胞，樹状細胞，B細胞，T細胞およびマクロファージの細胞膜上に認められる[60)70)]．

NKT細胞は活性化した後，自然免疫に関わるNK細胞[71)]や獲得免疫に関わるT，B細胞の活性化を引き起こすことによって[49)72)～74)]，様々な病原体に対する生体防御機構としての重要な役割を果たしている[75)76)]．NKT