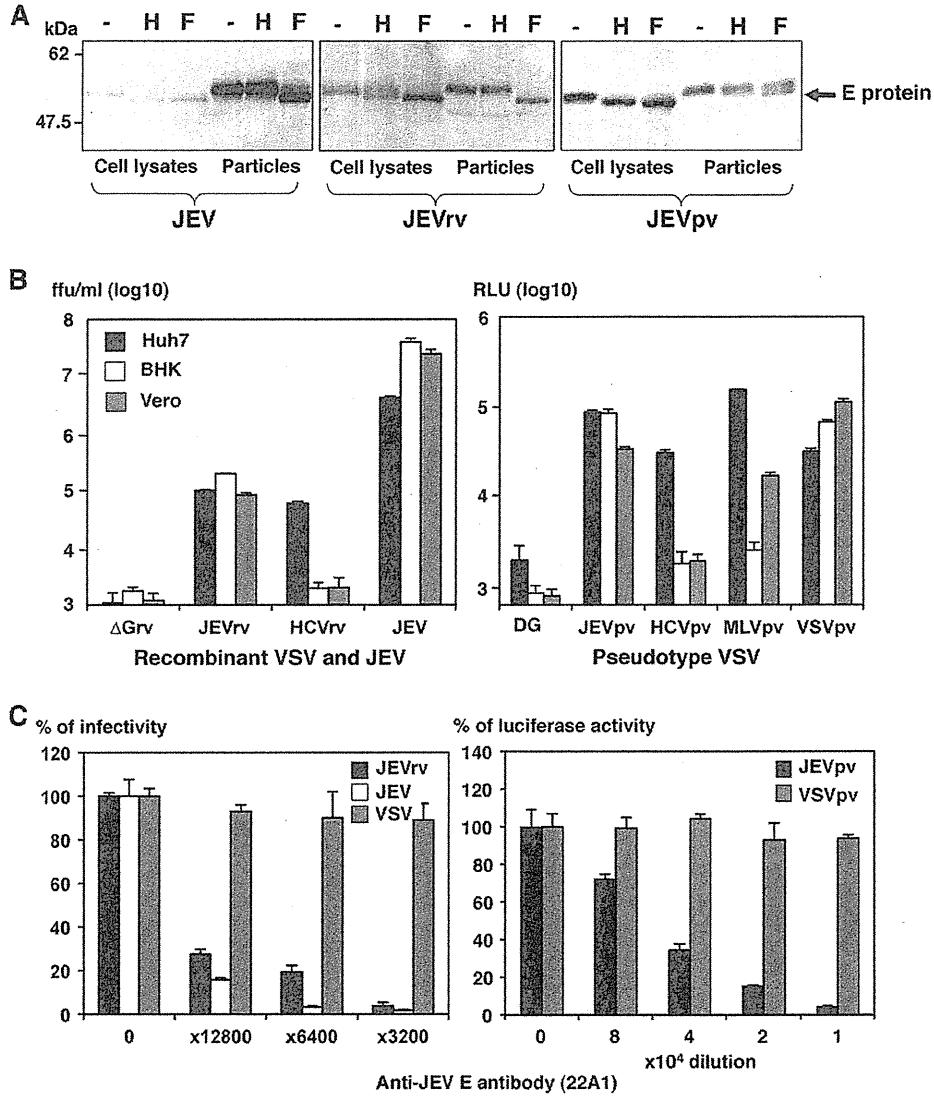

FIG. 2. Characterization of JEVrv and JEVpv. (A) JEV E proteins expressed in cells incorporated into the viral particles were treated with endoglycosidase H (H) or peptide-N-glycosidase F (F) and examined by immunoblotting using anti-E polyclonal antibody. "-" indicates an untreated sample. (B) Infectivities of recombinant viruses (left panel) and pseudotype viruses (right panel) were determined in Huh7, BHK, and Vero cells by a focus-forming assay and measurement of luciferase activity (RLU), respectively. VSV without envelope (ΔG) was used as a negative control. ffu, focus-forming units. (C) Neutralization of JEVrv (left panel) or JEVpv (right panel) infection by anti-E polyclonal antibody. Viruses were incubated with the indicated dilution of antibody for 1 h at room temperature and inoculated into Huh7 cells. Residual infectivities are expressed as percentages. VSV and VSVpv were used as controls. The results shown are from three independent assays, with error bars representing standard deviations.

and Vero cells, as previously reported (1). Although JEVpv and JEVrv generated in 293T cells were also infectious, these viruses were slightly more infective when generated in Huh7 cells, even though the efficiency of transfection of the expression plasmids into 293T cells was higher than that of transfection into Huh7 cells (data not shown). To determine the specificity of infection of JEVpv, JEVrv, and JEV, a neutralization assay was performed by using anti-E antibody (22A1). The infectivities of JEVpv and JEVrv but not of VSVpv and VSV for Huh7 cells were clearly inhibited by anti-E antibody in a dose-dependent manner (Fig. 2C). These results suggest that the JEVrv and JEVpv generated in this study had characteristics comparable to those of authentic JEV.

Entry pathways of JEVpv. Previous studies showed that JEV infection was inhibited by treatment with inhibitors of vacuolar acidification, such as ammonium chloride, concanamycin A, and bafilomycin A_1, suggesting that JEV enters target cells via pH-dependent endocytosis (30). Other flaviviruses, including WNV, DENV, and HCV, exhibit similar entry mechanisms (18, 45). To compare the entry pathway of JEV with those of other viruses, Huh7 cells were pretreated with various concentrations of bafilomycin A_1 and then the cells were inoculated

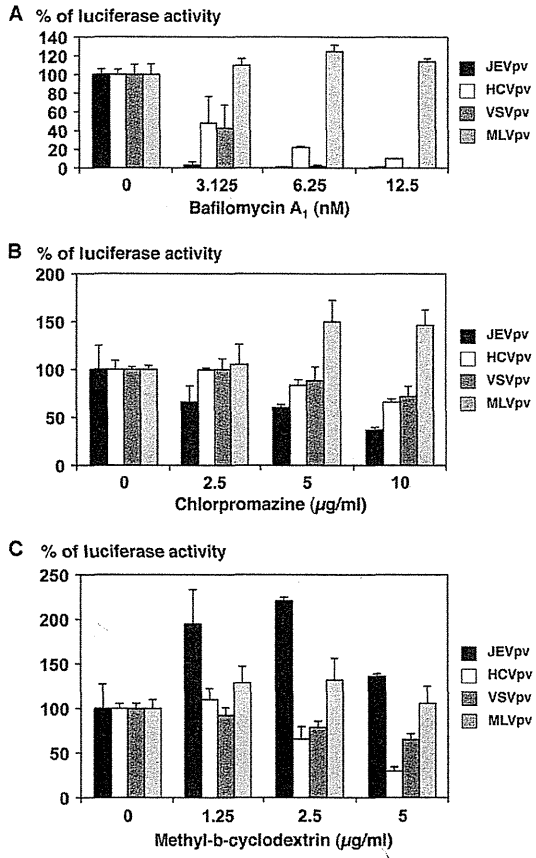

FIG. 3. Entry pathways of the pseudotype VSVs. Huh7 cells were pretreated with various concentrations of bafilomycin A_1 (A), chlorpromazine (B), or methyl-β-cyclodextrin (C) for 1 h and inoculated with the pseudotype viruses, JEVpv, HCVpv, VSVpv, and MLVpv. Luciferase activities were determined at 24 h postinfection. The results shown are from three independent assays, with error bars representing standard deviations.

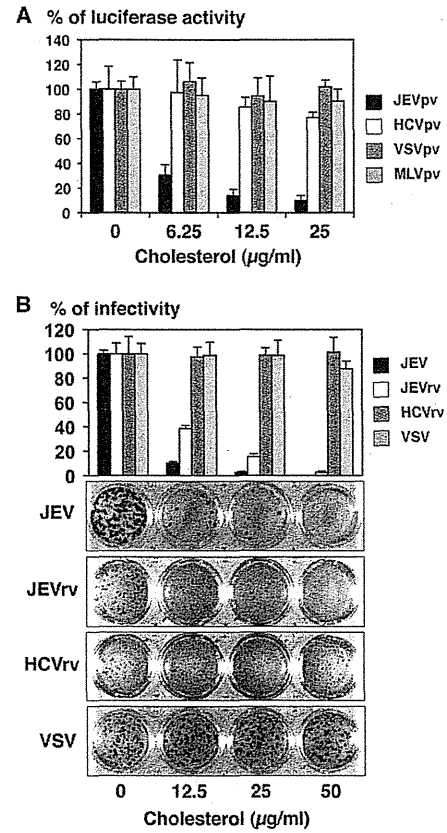

FIG. 4. Effects of cholesterol on infection with recombinant and pseudotype VSVs. (A) The pseudotype viruses were incubated with various concentrations of cholesterol for 1 h at room temperature and inoculated into Huh7 cells, and luciferase activities were determined at 24 h postinfection. (B) JEV, JEVrv, HCVrv, and VSV were incubated with various concentrations of cholesterol for 1 h at room temperature and inoculated into Huh7 cells, and residual infectivities were determined by focus-forming assay in a culture medium containing 1% methylcellulose at 48 h postinfection for JEV, JEVrv, and HCVrv and at 24 h postinfection for VSV. Foci of infected cells were detected by immunohistochemical staining (lower panel). The rate of focus formation of the viruses was analyzed by counting foci. The results shown are from three independent assays, with error bars representing standard deviations.

with JEVpv, HCVpv, VSVpv, and MLVpv (Fig. 3A). As expected, bafilomycin A_1 treatment did not affect the infectivity of MLVpv-bearing envelope proteins of MLV, which enters cells through a pH-independent direct fusion of the viral membrane and plasma membrane. In contrast, infections with HCVpv and VSVpv, which enter cells through pH-dependent endocytosis, were inhibited by treatment with bafilomycin A_1 in a dose-dependent manner. Similarly, infection with JEVpv was clearly inhibited by treatment with bafilomycin A_1 in a dose-dependent manner, suggesting that JEVpv enters cells through pH-dependent endocytosis, as seen in JEV infection.

To further examine the entry pathway of JEVpv, Huh7 cells were pretreated with various concentrations of chlorpromazine, an inhibitor of clathrin-mediated endocytosis, or MβCD, an inhibitor of caveolar/raft-mediated endocytosis, and infected with the pseudotype viruses. The infectivity of MLVpv was not affected by the treatment with either chlorpromazine or MβCD, as we expected. Treatment of cells with chlorpromazine slightly reduced the infectivity of JEVpv, HCVpv, and VSVpv in a dose-dependent manner (Fig. 3B), whereas treatment of cells with MβCD reduced the infectivity of HCVpv and VSVpv but increased the infectivity of JEVpv (Fig. 3C). These results suggest that JEVpv enters cells via clathrin-mediated endocytosis, as previously reported for infection with JEV (30), and that caveola/raft plays a different role in the entry of JEV than in the entry of HCV and VSV.

Effects of cholesterol on the entry and egress of JEV. Recently it was shown that entry of flaviviruses, including JEV and DENV, was drastically inhibited by treatment of the particles with cholesterol (20). To examine the effect of cholesterol on entry of JEV, the pseudotype viruses were inoculated into Huh7 cells after treatment with various concentrations of cholesterol. The infectivity of JEVpv but not that of HCVpv, VSVpv, or MLVpv was severely impaired by treatment with cholesterol in a dose-dependent manner (Fig. 4A). Next, to examine the effect of cholesterol on the propagation of JEV, the recombinant viruses were inoculated into Huh7 cells after

treatment with various concentrations of cholesterol. Infectivities of JEV and JEVrv but not those of VSV and HCVrv were inhibited by the treatment with cholesterol (Fig. 4B, upper panel). Suppression of the propagation of JEV and JEVrv was further confirmed by a focus-forming assay (Fig. 4B, lower panels). These results confirmed that JEV entry was suppressed by cholesterol, as previously reported (20), and raise the possibility that cholesterol participates not only in entry via the E protein but also in the assembly of the E protein. These data also support the notion that JEVpv and JEVrv are comparable to JEV in terms of the properties of the E protein involved in the entry and egress processes.

Effects of SMase on infection with JEVpv, JEVrv, and JEV. Because infection with enveloped viruses was initiated by the interaction of viral and host membrane lipids, we next examined the involvement of membrane lipids in the entry of JEV. Sphingolipid is a major component of eukaryotic lipid membranes, and sphingomyelin is one of the most abundant sphingolipids, with a wide presence across the cell membrane. SMase is known to cleave sphingomyelin, yielding phosphorylcholine and ceramide. To examine the effect of SMase on viral infection, cells were infected with viruses after treatment with various concentrations of SMase, and the infectivities of the viruses were assessed by the luciferase or focus-forming assay. Infection with JEVpv was drastically enhanced by SMase treatment of Huh7 cells, whereas such treatment exhibited no effect on infection with VSVpv and MLVpv and suppressed HCVpv infection (Fig. 5A). The enhancement of JEVpv infection by SMase treatment was also observed in other cell lines, including BHK and Vero cells (data not shown). Although the effect was not as evident as in JEVpv infection, SMase treatment exhibited a slight but substantial enhancement of the infectivity of JEV and JEVrv in Huh7 cells, in contrast to having no effect on VSV infection and a suppressive effect on HCVrv infection (Fig. 5B). The difference in the magnitude of enhancement of infectivity by treatment with SMase between infection with JEVpv and that with JEV or JEVrv might be attributable to the difference in the viral systems based on pseudotype (JEVpv) and replication-competent (JEV and JEVrv) viruses, which allow single and multiple rounds of infection, respectively. The effects of SMase may be more critical for the entry step than for other, later steps of infection. Suppression of HCVpv and HCVrv infection by treatment with SMase was consistent with previously reported data on infection of HCV-pseudotyped retroviral particles (HCVpp) and JFH1 virus (48).

Next, we examined the effect of SMase on the viral particles. Treatment of pseudotype particles of JEVpv, VSVpv, and MLVpv with various concentrations of SMase had no significant effect on their infectivity for Huh7 cells (Fig. 5C), whereas the infectivity of HCVpv particles was impaired by the treatment in a dose-dependent manner, as reported previously (1), suggesting that SMase treatment enhances the infectivity of JEVpv by modifying the molecules on target cells rather than the molecules on viral particles. To further determine the involvement of SMase in infection of JEVpv, cells were pretreated with various concentrations of amitriptyline, an inhibitor of acid SMase. The infectivity of JEVpv but not that of other viruses was decreased by the treatment with amitriptyline in a dose-dependent manner (Fig. 5D). A similar effect was observed with treatment with another SMase inhibitor, imipramine (data not shown). Collectively, these results suggest that entry of JEV into the target cells is enhanced by SMase treatment, which modifies the cell surface sphingolipids into a more competent state for interaction with the JEV envelope protein, thereby enabling its entry.

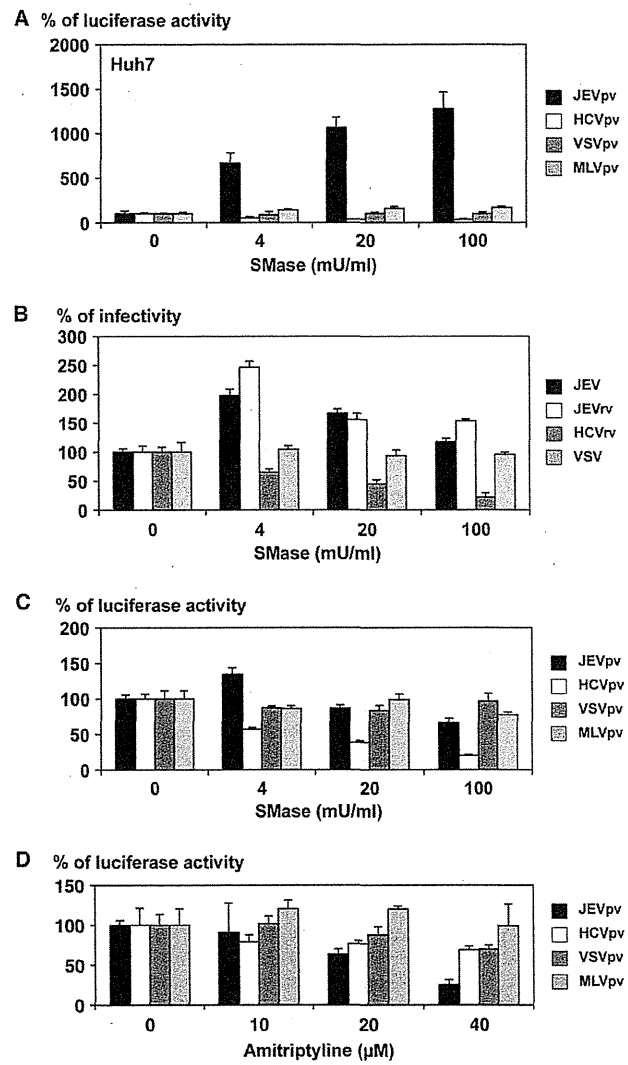

FIG. 5. Effects of SMase and amitriptyline treatment of cells on infection with pseudotype and recombinant VSVs. Huh7 cells were pretreated with various concentrations of SMase for 1 h, and then pseudotype viruses (A) or recombinant viruses (B) were inoculated. The infectivities were determined by luciferase activity measurement or focus-forming assay, and changes in infectivities are expressed as percentages. (C) The purified pseudotype particles were treated with various concentrations of SMase for 1 h and inoculated into Huh7 cells after removal of SMase by ultracentrifugation. Infectivities were determined at 24 h postinfection by measuring luciferase activity, and changes in infectivities are expressed as percentages. (D) Huh7 cells were pretreated with various concentrations of amitriptyline, an inhibitor for the acid SMase, for 1 h, and then pseudotype viruses were inoculated. Infectivities were determined at 24 h postinfection by measuring luciferase activity, and changes in infectivities are expressed as percentages. The results shown are from three independent assays, with error bars representing standard deviations.

Effects of SMase on propagation of JEVrv and JEV. We next examined the effects of SMase on the propagation of JEV. Recombinant VSV is capable of replicating by using the VSV genome and producing infectious particles bearing a foreign envelope protein encoded in place of the original G protein, and thus, it is feasible to assess the efficiency of not only entry but also egress of the recombinant viruses possessing foreign envelope genes of different origins, irrespective of their replication efficiency within the target cells. To examine the effects of SMase on viral propagation, cells were treated with various concentrations of the enzyme, inoculated with the recombinant viruses, and then cultured for up to 48 h in the presence of SMase. Production of JEVrv was dramatically enhanced by cultivation in the presence of SMase, in contrast to the suppression of HCVrv propagation (Fig. 6A). Although the effect of SMase treatment on the production of JEV was not as great as that seen in JEVrv propagation, treatment with SMase resulted in a substantial enhancement of JEV but not of VSV propagation (Fig. 6B). These results suggest that SMase treatment induces robust propagation of JEVrv mainly through enhancement of the entry step although also partly through enhancement of the egress step.

Involvement of ceramide in infection with JEV. Because treatment of cells with SMase induces production of ceramide, we next examined the effect of ceramide on the infectivity of the viruses. Treatment of the pseudotype particles with C_6-ceramide inhibited the infectivity of JEVpv for Huh7 cells in a dose-dependent manner, whereas no clear reduction of infectivity was observed with treatment of HCVpv, VSVpv, and MLVpv with ceramide (Fig. 7A). In contrast, treatment of the pseudotype particles with sphingomyelin, which is a substrate for SMase and is catalyzed into ceramide, did not affect the infectivity of the viruses, suggesting that the enhancement of infectivity of JEVpv by treatment with SMase was due to the generation of ceramide. Propagation of JEV but not of VSV was also suppressed by treatment of the viral particles with C_6-ceramide in a dose-dependent manner (Fig. 7B). Finally, to confirm the interaction of the JEV E protein with ceramide, purified JEV and JEVrv particles were incubated with biotin-ceramide and streptavidin-Sepharose 4B and examined by pull-down assay (Fig. 7C). The E proteins of both JEV and JEVrv were precipitated with the ceramide beads. These results indicate that the interaction of the JEV E protein with ceramide plays a crucial role in the entry of JEV.

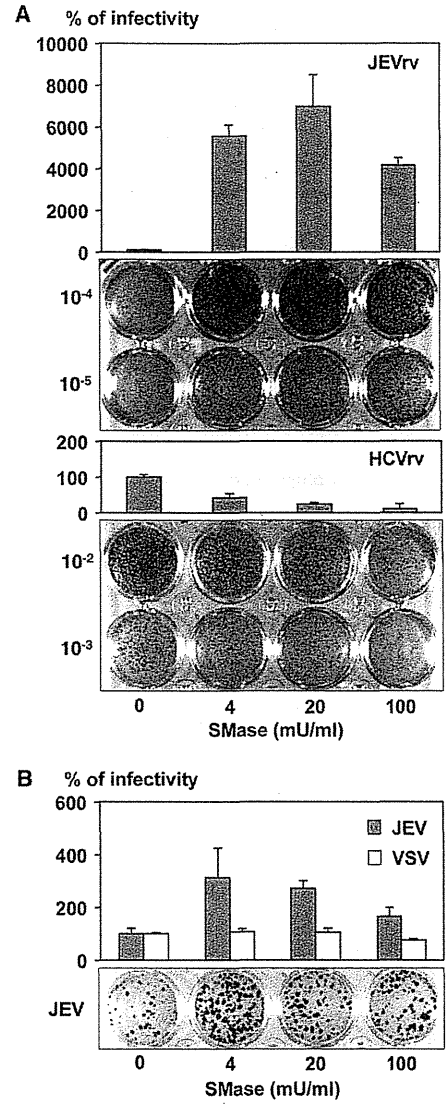

FIG. 6. Effects of SMase on the propagation of JEVrv and JEV. Huh7 cells were pretreated with various concentrations of SMase for 1 h and inoculated with JEVrv or HCVrv (A) or JEV or VSV (B), and infectivities were determined by focus-forming assay in a culture medium containing 1% methylcellulose at 48 h after infection with JEVrv, HCVrv, and JEV and at 24 h after infection with VSV. Titers were determined by counts of foci detected by immunohistochemical staining (lower panels). The results shown are from three independent assays, with error bars representing standard deviations.

DISCUSSION

Ceramide has been shown to play a crucial role in various cell signaling pathways through the clustering and activation of the receptor molecules in lipid rafts. Although the generation of ceramide inhibits the infectivity of HIV and HCV by the rearrangement of the entry receptor molecules (7, 48), rhinovirus and Sindbis virus generate ceramide by activating SMase for their entry and cell survival, respectively (10, 15). In this study, we demonstrated for the first time that ceramide plays crucial roles not only in the entry pathway of JEV but also in the egress through a direct interaction with the E envelope proteins.

To examine the roles of the E protein in the infectivity of JEV, we employed pseudotype and recombinant VSVs bearing JEV envelope proteins as surrogate systems in addition to authentic JEV. VSV assembles and buds from the plasma membrane, and therefore the surrogate viruses bearing the foreign envelope proteins being expressed on the plasma membrane exhibited more-efficient incorporation of the envelope proteins. Although the E protein of JEV, as well as that of other flaviviruses, including HCV, is mainly retained on the endoplasmic reticulum (ER) membrane, the E protein was incorporated into JEVpv and JEVrv particles and exhibited infectivity comparable to that of authentic JEV. Further studies are needed to clarify the mechanisms of incorporation of

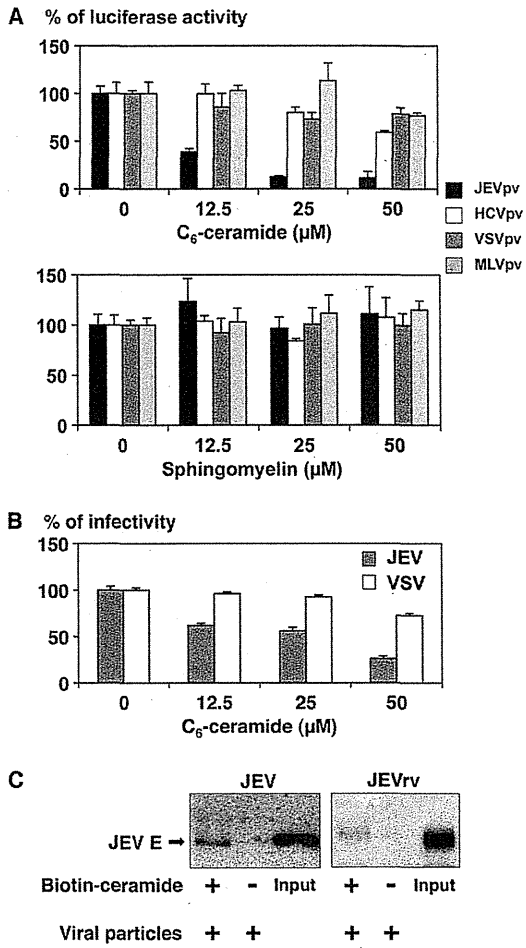

FIG. 7. Involvement of ceramide in infection with JEV. (A) Effects of C_6-ceramide or sphingomyelin in infection with JEVpv. Purified pseudotype viruses were pretreated with various concentrations of C_6-ceramide (upper) or sphingomyelin (lower) for 1 h and then inoculated into Huh7 cells. The infectivities were determined at 24 h postinfection by luciferase activity, and changes in infectivities are expressed as percentages. (B) Effects of C_6-ceramide in infection of JEVrv and JEV. JEV and VSV were pretreated with various concentrations of C_6-ceramide for 1 h, and then the viruses were inoculated into Huh7 cells. At 24 h postinfection, the infectivities were determined by focus-forming assay. (C) Binding of JEV and JEVrv to ceramide beads. Purified viruses were preincubated with (+) or without (−) biotin-ceramide resolved in DMSO and streptavidin-Sepharose 4B. After washing, residual pellets were analyzed by immunoblotting. Inputs are purified viruses. The results shown are from three independent assays, with error bars representing standard deviations.

the foreign envelope proteins on the ER membrane into VSV particles. In general, glycoproteins are modified into the complex type during the translocation from the ER to the Golgi apparatus. Although the JEV E glycoproteins were modified mainly into the high-mannose type in cells infected with JEVpv, JEVrv, or JEV, viruses possessing the E proteins were modified into the complex type within the particles. These results suggest that the E proteins of JEV and the surrogate viral particles are modified into the complex type after budding into the ER lumen during translocation into the Golgi apparatus. Recently assembly of DENV in the ER was revealed by three-dimensional architecture using electron tomography (49).

A number of viruses utilize cholesterol-rich membrane microdomains or lipid rafts for their entry, assembly, or egress processes (5). Cholesterol-rich membrane microdomains have been shown to be required for the entry but not for the replication of WNV through cholesterol depletion by treatment with MβCD (27). Entry of HCV was also shown to be partially required for cellular cholesterol (1, 16), which is consistent with the present data that infection with HCVpv was partially inhibited by treatment of cells with MβCD. Lee et al. recently reported that the infectivity of JEV, especially the replication step, was inhibited by treatment of cells with MβCD or the cholesterol chelation antibiotic filipin III (20). Furthermore, treatment of the viral particles with cholesterol inhibited the infectivity of JEV, in contrast to the enhancement of the infectivity of Sindbis virus by the same treatment (20, 22). Our data also indicated that the infectivity of JEVpv and JEVrv, as well as that of JEV, was completely inhibited by treatment of the particles with cholesterol in a dose-dependent manner, supporting the notion that the presence of an abundant amount of cholesterol increases the rigidity of the E protein of JEV particles and inhibits the membrane fusion event, as suggested by Lee et al. (20).

According to the current models, SMase alters the biophysical properties of the membrane bilayer by generating ceramide through the hydrolysis of sphingomyelin. Genetic disorders of SMase or ceramide metabolism are critically involved in human genetic diseases, such as Niemann-Pick disease (37) and Wilson's disease (19). *In vivo* studies of the function of SMase or ceramide in infections with pathogens are accumulating (9, 44, 46), and acid SMase-deficient mice have been shown to be unable to eliminate the pathogens because of failure to undergo apoptosis or phagolysosomal fusion, ultimately a massive release of cytokines and death by sepsis. It has recently been shown that acid SMase is a key regulator of cytotoxic granule secretion by primary T lymphocytes (13). The reduction of the cytolytic activity of $CD8^+$ cytotoxic T lymphocytes in acid SMase-deficient mice resulted in a significantly delayed clearance of lymphocytic choriomeningitis virus infection. Recently it was shown that entry of HCV is inhibited by SMase treatment through the downregulation of CD81, a major receptor of HCV, because enrichment of ceramide on the plasma membrane induces internalization of CD81 (48). HIV infection is also inhibited by ceramide enrichment through a restriction of the lateral diffusion of CD4 (6). Sindbis virus and rhinovirus activate the SMase and induce generation of ceramide in the endosomal membrane. Inhibition of SMase by genetic manipulation or pharmacological agents prevents infection with rhinoviruses, suggesting that SMase and ceramide-enriched membrane platforms play an important role in viral infection (10).

In this study, we have shown that entry of JEVpv, JEVrv, and JEV was specifically enhanced by treatment of cells with SMase. Treatment of cells with amitriptyline, an inhibitor interfering with the binding of SMase to the lipid bilayer, impaired the uptake of rhinovirus (10) and *Neisseria gonorrhoeae* (8). The entry of JEVpv was also inhibited by treatment with the inhibitor. Furthermore, the infections of JEVpv and JEV were inhibited by treatment with C_6-ceramide but not by treat-

ment with sphingomyelin, and JEV and JEVrv were coprecipitated with the ceramide beads, suggesting that the interaction of ceramide with the JEV E protein plays a crucial role in the early steps of infection. Ceramide is known to bind to the ceramide transport protein (CERT), which transports ceramide from the ER to the Golgi apparatus (12), and thus, it might be feasible to speculate that CERT participates in the translocation or maturation of the JEV E protein. Further studies are needed to clarify the interaction among ceramide, CERT, and the JEV E protein. Recently Aizaki et al. reported that the infectivity of HCV particles was decreased by treatment with MβCD or SMase, suggesting that cholesterols or sphingolipids incorporated into the virions are important for the infectivity of HCV (1). In this study, SMase treatment of HCVpv particles but not of JEVpv particles reduced infectivity, suggesting that incorporation of cholesterols and sphingolipids into the viral particles was different among flaviviruses.

The discrepancy between the drastic increase in the production of infectious particles of JEVrv and the marginal increase in that for JEV induced by SMase treatment in ceramide-enriched cells may indicate that ceramide enrichment enhances the entry and egress steps but negatively regulates genomic replication of JEV. Previously it was reported that digestion of sphingomyelin by SMase induces cholesterol redistribution (32), an increase in intracellular cholesterol esterification (4), and a decrease in cholesterol biosynthesis (39). Furthermore, ceramide has been shown to selectively displace cholesterol from lipid rafts and decrease the association of the cholesterol binding protein caveolin-1 (28, 50). Although we have not determined the cholesterol composition of the membranes of cells treated with SMase, cholesterol depletion induced by SMase treatment may also participate in the enhancement of JEV entry.

JEV initiates infection by interacting with receptor and/or coreceptor molecule(s), probably in cooperation with ceramide located in the ceramide-enriched platforms. The ceramide-enriched membrane domains facilitate signal transduction through reorganization and clustering of cell surface receptor molecules. Although the entry receptor(s) of JEV has not been well characterized yet, modification of the distribution, organization, and steric conformation of the receptor molecule(s) by treatment with SMase may facilitate entry of JEV. Generation of ceramide by SMase treatment has been shown to promote vesicular fusion processes and fusion of phagosomes, thereby engulfing bacteria with late endosomes and resulting in efficient intracellular bacterial killing (46).

In conclusion, we have demonstrated that the entry and egress processes of JEV were enhanced by treatment with SMase by using pseudotype and recombinant VSVs. The interaction of cellular ceramide and the E glycoproteins facilitates infection and propagation of JEV. Modification of sphingolipids on the plasma membrane of the target cells might be a novel target for the development of antivirals against JEV infection.

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Factors affecting efficacy in patients with genotype 2 chronic hepatitis C treated by pegylated interferon alpha-2b and ribavirin: reducing drug doses has no impact on rapid and sustained virological responses

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SUMMARY. Reducing the dose of drug affects treatment efficacy in pegylated interferon (Peg-IFN) and ribavirin combination therapy for patients with hepatitis C virus (HCV) genotype 1. The aim of this study was to investigate the impact of drug exposure, as well as the baseline factors and the virological response on the treatment efficacy for genotype 2 patients. Two-hundred and fifty patients with genotype 2 HCV who were to undergo combination therapy for 24 weeks were included in the study, and 213 completed the treatment. Significantly more patients who achieved a rapid virological response (RVR), defined as HCV RNA negativity at week 4, achieved a sustained virological response (SVR) (92%, 122/133) compared with patients who failed to achieve RVR (48%, 38/80) ($P < 0.0001$). Multivariate logistic-regression analysis showed that only platelet counts [odds ratio (OR), 1.68; confidence interval (CI), 1.002–1.139] and RVR (OR, 11.251; CI, 5.184–24.419) were independently associated with SVR, with no correlation being found for the mean dose of Peg-IFN and ribavirin for RVR and SVR. Furthermore, in the stratification analysis of the timing of viral clearance, neither mean dose of Peg-IFN ($P = 0.795$) nor ribavirin ($P = 0.649$) affected SVR in each group. Among the patients with RVR, the lowest dose group of Peg-IFN (0.77 ± 0.10 μg/kg/week) and ribavirin (6.9 ± 0.90 mg/kg/day) showed 100% and 94% of SVR. Hence, RVR served as an important treatment predictor, and drug exposure had no impact on both SVR and RVR in combination therapy for genotype 2 patients.

Keywords: chronic hepatitis C, drug exposure, genotype 2, peginterferon and ribavirin combination therapy.

INTRODUCTION

The current standard of care for chronic hepatitis C (CHC) patients consists of combination therapy using pegylated interferon (Peg-IFN) and ribavirin [1–3]. Large, randomized clinical trials have demonstrated that 42–52% of hepatitis C virus (HCV) genotype 1 'difficult-to-treat' patients achieved sustained virological response (SVR), whereas 76–84% of HCV genotype 2 or 3 infected patients treated with Peg-IFN and ribavirin achieved SVR [4–6]. It also has been shown that in HCV genotype 2 and 3 infected patients, 24-week treatment regimens are just as effective as 48-week regimens [6,7]. Therefore, current guidelines recommend a 24-week treatment for these patients in contrast to 48 weeks for genotype 1 patients [1–3]. However, as side effects are common and treatment is expensive for this therapy, it would be ideal to be able to further reduce the total amount of drug medication

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CHC, chronic hepatitis C; c-EVR, complete early virological response; ETR, end of treatment response; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; IFN, interferon; NPV, negative predictive value; Peg-IFN, pegylated interferon; RVR, rapid virological response; SVR, sustained virological response.

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without loss of treatment efficacy for genotype 2 and 3 patients.

In HCV genotype 1 patients, reducing drug doses affects treatment efficacy. In our investigation of HCV genotype 1 patients, the rate of complete early virological response (c-EVR), defined as HCV RNA negativity at week 12, was affected by the mean dose of Peg-IFN during the first 12 weeks dose-dependently ($P < 0.0001$) [8]. Furthermore, we showed that only 4% relapse was found in patients given ≥12 mg/kg/day of ribavirin among those with c-EVR, and the relapse rate showed a decline in relation to the increase in the dose of ribavirin ($P = 0.0002$) [9]. On the contrary, it remains to be determined whether treatment efficacy can be preserved by further reducing both drug doses in genotype 2 and 3 patients. Because lower doses are expected to cause fewer adverse effects, it is important to find whether reduced drug doses can be used while retaining efficacy.

In the present study, we retrospectively evaluated the efficacy of Peg-IFN alpha-2b and ribavirin combination therapy for 24 weeks in patients infected with HCV genotype 2 and analysed the factors that affected the treatment efficacy, with particular interests in the drug impact of Peg-IFN and ribavirin.

PATIENTS AND METHODS

Patient selection and study design

Patients considered to be eligible for this study were those infected with HCV genotype 2 who underwent Peg-IFN alpha-2b (Schering-Plough K.K., Tokyo, Japan) and ribavirin (Schering-Plough K.K.) combination therapy from December 2005 to July 2007 at 29 medical institutions taking part in the Osaka Liver Forum and had completed the 24-week observation after a clinical course of 24 weeks. Patients with the following criteria were excluded: hepatitis B virus or human immunodeficiency virus coinfection, decompensated liver disease, severe cardiac, renal, haematological or chronic pulmonary disease, poorly controlled psychiatric disease, poorly controlled diabetes and immunologically mediated disease. Liver biopsy had been performed within 24 months prior to the treatment, and histological results were classified according to the METAVIR scoring system [10].

Written informed consent was obtained from each patient, and the study protocol was reviewed and approved according to the ethical guidelines of the 1975 Declaration of Helsinki by institutional review boards at the respective sites.

Patients were treated with Peg-IFN alpha-2b plus ribavirin for the duration of the study of 24 weeks. Peg-IFN alpha-2b and ribavirin dosages were based on body weight according to the manufacturer's instructions: Peg-IFN alpha-2b was given subcutaneously weekly (45 kg or less, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; 91 kg or more, 150 μg/dose), and ribavirin was given orally daily (60 kg or less, 600 mg/day; 61–80 kg, 800 mg/day; 81 kg or more, 1000 mg/day). The drug doses were also modified based on the manufacturer's instructions according to the intensity of the haematologic adverse effects.

Virological tests

Serum HCV RNA level was quantified by PCR assay (COBAS Amplicor HCV Test v2.0, Chugai-Roche Diagnostics, Tokyo, Japan), with a sensitivity limit of 5000 IU/mL and a dynamic range from 5000 to 5 000 000 IU/mL [11].

Serum HCV RNA was assessed by qualitative PCR assay (COBAS Amplicor HCV Monitor Test v2.0, Chugai-Roche Diagnostics), with a detection limit of 50 IU/mL [12].

Assessment of efficacy

Serum HCV RNA (qualitatively or quantitatively) was measured at weeks 4, 8, 12 and 24 during treatment and after 24 weeks of follow-up without treatment. Patients were classified as having a rapid virological response (RVR) if serum HCV RNA was undetectable (<50 IU/mL) at week 4 and at the end of treatment response (ETR) at week 24 of treatment. SVR was defined as undetectable HCV RNA at week 24 after treatment. Patients with an ETR who seroreverted to HCV RNA during follow-up were classified as relapsers.

Drug exposure

The amounts of Peg-IFN alpha-2b and ribavirin actually taken by each patient during the treatment period were evaluated by reviewing the medical records. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline; Peg-IFN alpha-2b expressed as μg/kg/week and ribavirin as mg/kg/day.

Data collection

The medical records were retrospectively reviewed and the factors necessary for this examination were extracted: age, sex, body weight, body mass index (BMI), basic laboratory assessments, liver histology, quantitative and qualitative HCV RNA, dose of Peg-IFN alpha-2b and ribavirin received at each administration, and the response to treatment.

Statistical analysis

This study was a retrospective study and, for treatment results and the analysis of related factors, analysis was carried out only for cases in which the treatment had been completed (per-protocol analysis). Continuous variables are reported as the mean with standard deviation (SD) or

median level, while categorical variables are shown as the count and proportion. In univariate analysis, the Mann–Whitney *U*-test was used to analyse continuous variables, while chi-squared and Fisher's exact tests were used for analysis of categorical data. Variables with $P < 0.05$ at univariate analysis were retained for the multivariate logistic-regression analysis. Stepwise and multivariate logistic-regression models were used to explore the independent factors that could be used to predict a virological response. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. For all tests, two-sided *P*-values were calculated and the results were considered statistically significant if $P < 0.05$. Statistical analysis was performed using the SPSS program for Windows, version 15.0J (SPSS, Chicago, IL, USA).

RESULTS

The baseline characteristics for the total cohort are shown in Table 1. Most of the patients were female (56%) with a mean age of 54 years. Seventy per cent of the patients were treatment naïve. Of the 250 patients, liver biopsies were performed for 174 patients, and 18 of them had advanced fibrosis (F 3–4).

Of the total of 250 patients, 37 (15%) were withdrawn from treatment because of adverse events: decreased haemoglobin ($n = 10$), psychiatric problems including depression ($n = 9$), fatigue ($n = 3$), thrombocytopenia, neutropenia, pyrexia, rash, cerebral haemorrhage, bleeding of ocular fundus, dyspnea, dizziness, jaundice, transaminase rise, gastrointestinal symptoms ($n = 1$) and other adverse events ($n = 4$). Eight of these patients who discontinued treatment prematurely had SVR (8/37; 22%).

Drug adherence

Seventy-nine of the 213 patients (37%) required dose reduction of Peg-IFN alpha-2b, 99 (46%) of ribavirin because of adverse events (not including patients who later discontinued treatment because of adverse event). Neutropenia (24/79; 30%) and thrombocytopenia (24/79; 30%) were the most common adverse events for dose reduction of Peg-IFN alpha-2b, and decreased haemoglobin (82/99; 83%) for that of ribavirin.

Virological response

Of the 213 patients who completed 24 weeks of treatment and 24 weeks of follow-up, 160 (75%) patients were clear of HCV RNA at week 4, 191 (90%) at week 8, 196 (92%) at week 12. ETR was observed for 195 (92%), and SVR for 160 (75%). The relapse rate was 18% (35/195).

Virological response according to the timing of viral clearance

Positive and negative prediction of sustained virological response according to the timing of viral clearance

We examined SVR rates according to the timing of viral clearance for the case in which HCV RNA was cleared during the treatment (Fig. 1a). The SVR rate was 92% (122/133) for patients clear of HCV RNA until week 4, 64% (37/58) from week 5 until week 8, 20% (1/5) from week 9 until

Table 1 Baseline demographic and viral characteristics of patients

Number of cases	250	
Age (years)*	54.0 ± 12.4	(22–76)
Sex (male/female)	110/140	
Body weight (kg)*	60.3 ± 11.7	(39–99)
Body mass index (kg/m^2)*	23.1 ± 3.2	(16–35)
Past IFN therapy (naïve/experienced)†	175/70	
HCV RNA (KIU/mL)‡	1700	(4–5000 <)
Fibrosis (0/1/2/3/4)§	18/98/40/14/4	
Activity (0/1/2/3)§	15/81/70/8	
White blood cells ($/mm^3$)*	5210 ± 1,750	(2100–13 870)
Neutrophils ($/mm^3$)*	2700 ± 1,250	(590–9020)
Red blood cells ($\times 10^4/mm^3$)*	436 ± 48	(307–554)
Haemoglobin (g/dL)*	13.9 ± 1.4	(10–18)
Platelets ($\times 10^4/mm^3$)*	18.3 ± 6.4	(4–41)
ALT (IU/L)*	79 ± 77	(13–581)
γ-GTP (U/L)*	56 ± 65	(7–479)
Creatinine(mg/dL)*	0.7 ± 0.1	(0.4–1.1)

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase. *Values expressed as mean ± SD (range), †interferon treatment history was not known for five patients, ‡values expressed as median (range), §data for 76 patients are missing.

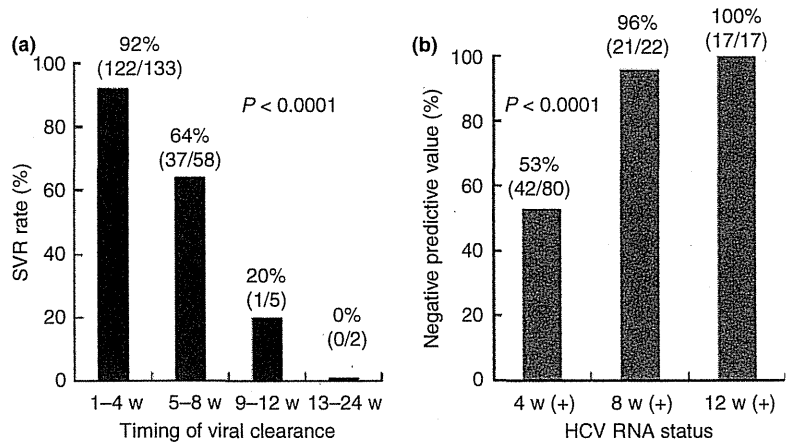

Fig. 1 (a) SVR rates according to timing of viral clearance. The number above each bar shows the percentage, and the numbers inside parentheses show the number of patients showing responses over the total number in the subgroup. The timing of viral clearance was time-dependently correlated with SVR ($P < 0.0001$). (b) Negative predictive values according to time of HCV RNA positivity. The number above each bar shows the percentage, and the numbers inside parentheses show the number of patients showing responses over the total number in the subgroup. The time of HCV RNA positivity was time-dependently correlated with NPV ($P < 0.0001$).

week 12 and 0% (0/2) from week 13 until week 24. The Mantel–Haenszel chi-square test showed that SVR rates were diminished with a delay in the timing of viral clearance becoming late ($P < 0.0001$). Significantly, more patients who attained RVR achieved final SVR (92%, 122/133) than patients who failed to attain RVR (48%, 38/80; $P < 0.0001$).

Next, we examined the negative predictive value (NPV) for the proportion of patients with treatment failure among those with HCV RNA persistence at week 4, 8 and 12 (Fig. 1b). NPV was 53% at week 4, 96% at week 8 and 100% at week 12. Only one of the 22 patients with positive HCV RNA at week 8 reached SVR.

Predictors of sustained virological response

Both pretreatment and treatment factors that could be associated with the response to Peg-IFN and ribavirin combination therapy were compared between patients with and without SVR in Table 2. This univariate analysis showed that age ($P = 0.029$), baseline HCV RNA level ($P = 0.033$), past IFN treatment history ($P = 0.028$), platelets counts ($P = 0.020$) and having RVR ($P < 0.0001$) contributed to achievement of SVR. Factors that were significantly associated with SVR by univariate analysis were then analysed by multivariate logistic regression analysis. SVR was attained independent of high platelet counts [odds ratio (OR) 1.070, 95% confidence interval (CI) 1.003–1.140, $P = 0.040$] and having RVR (OR 11.526, 95% CI 5.317–24.984, $P < 0.0001$; Table 3). As for drug doses, the mean dose of Peg-IFN alpha-2b was 1.32 ± 0.27 μg/kg/week in patients with SVR and 1.27 ± 0.29 μg/kg/week in those without SVR ($P = 0.130$), while that of ribavirin was 10.2 ± 1.9 and 10.2 ± 2.0 mg/kg/day ($P = 0.949$), respectively. Thus, neither Peg-IFN nor ribavirin drug exposure during the full treatment period affected attainment of SVR.

Predictors of rapid virological response

To delineate features that might help identify patients most likely to reach RVR, we also analysed these factors because having RVR turned out to be one of the most powerful predictors of SVR attainment. By univariate and multivariate logistic-regression analyses, RVR was attained independent of younger age (OR 0.648, 95% CI 0.494–0.850, $P = 0.002$) and lower baseline HCV RNA level (OR 0.964, 95% CI 0.944–0.984, $P < 0.0001$; Tables 4 & 5). The mean dose of Peg-IFN alpha-2b during the first 4 weeks was 1.31 ± 0.27 μg/kg/week in patients with RVR and 1.31 ± 0.29 μg/kg/week in those without RVR ($P = 0.259$), that of ribavirin was 10.1 ± 1.8 mg/kg/day and 10.3 ± 2.1 mg/kg/day ($P = 0.637$), respectively. Thus, neither Peg-IFN nor ribavirin drug exposure during the first 4 weeks had an impact on attainment of RVR.

Virological response according to drug exposure and the timing of viral clearance

Impact of drug exposure on sustained virological response

To more closely evaluate the impact of drug exposure on virological response, we classified the average doses of both drugs into four categories (Peg-IFN alpha-2b: up to 0.9 μg/kg/week, from 0.9 to >1.2 μg/kg/week, from 1.2 to >1.5 μg/kg/week, from 1.5 μg/kg/week; ribavirin: up to

Table 2 Factors associated with SVR among patients who completed the treatment – univariate analysis

Factor	SVR (n = 160)	Non-SVR (n = 53)	P-value
Age (years)*	52.4 ± 12.6	56.9 ± 10.2	0.029
Sex (male/female)	66 / 94	26 / 27	0.202
Body weight (kg)*	59.5 ± 11.5	59.9 ± 12.5	0.896
Body mass index (kg/m^2)*	22.8 ± 3.1	22.8 ± 3.5	0.817
HCV RNA (KIU/mL)†	1170	1600	0.033
Past IFN therapy (naive/experienced)‡	116/41	31/22	0.028
Fibrosis (F 0–2/3–4)§	106/10	30/5	0.247
Activity (A 0–1/2–3)§	62/54	20/15	0.847
White blood cells (/mm^3)*	5260 ± 1680	4720 ± 1500	0.078
Neutrophils (/mm^3)*	2740 ± 1270	2420 ± 1020	0.186
Red blood cells (×10^4/mm^3)*	435 ± 44	437 ± 55	0.820
Haemoglobin (g/dL)*	13.9 ± 1.3	14.0 ± 1.5	0.441
Platelets (×10^4/mm^3)*	19.0 ± 6.0	16.5 ± 6.2	0.020
ALT (IU/L)*	86 ± 89	64 ± 45	0.514
γ-GTP (U/L)*	54 ± 67	58 ± 59	0.512
Creatinine (mg/dL)*	0.7 ± 0.1	0.7 ± 0.1	0.457
Mean Peg-IFN dose (μg/kg/week)*	1.32 ± 0.27	1.27 ± 0.29	0.130
Mean ribavirin dose (mg/kg/day)*	10.2 ± 1.9	10.2 ± 2.0	0.949
RVR (yes/no)	122/11	38/42	<0.0001

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; CI, confidence interval. *Values expressed as mean ± sd, †values expressed as median, ‡interferon treatment history was not known for three patients, §data for 62 patients are missing.

Table 3 Factors associated with SVR among patients who completed the treatment – multivariate analysis

Factor	Category	Odds ratio	95% CI	P-value
Age (years)	By 10	–	–	NS
HCV RNA (KIU/mL)	By 100 KIU/mL	–	–	NS
Platelets (×10^4/mm^3)	By 1 × 10^4/mm^3	1.068	1.002–1.139	0.045
Past IFN therapy	Naïve/experienced	–	–	NS
RVR	Yes/no	11.251	5.184–24.419	<0.0001

IFN, interferon; HCV, hepatitis C virus; CI, confidence interval.

8 mg/kg/day, from 8 to >10 mg/kg/day, from 10 to >12 mg/kg/day, from 12 mg/kg/day). SVR rates relative to the mean drug doses during the full treatment period and the timing of HCV RNA clearance are shown in Table 6. As also shown in Fig. 1a, the respective rates for SVR according to the timing of viral clearance were 92% in patients clear of HCV RNA until week 4, 64% from week 5 until week 8 and 14% from week 9 until week 24. On the contrary, according to mean drug doses, the respective rates for SVR were 89% (24/27), 73% (11/15), 79% (85/107) and 82% (40/49) in patients who received Peg-IFN up to 0.9 μg/kg/week, from 0.9 to >1.2 μg/kg/week, from 1.2 to >1.5 μg/kg/week and from 1.5 μg/kg/week, respectively, and 80% (24/30), 80% (40/50), 82% (68/83) and 79% (27/34) in patients who received ribavirin up to 8 mg/kg/day, from 8 to >10 mg/kg/day, from 10 to >12 mg/kg/day and from 12 mg/kg/day, respectively. If the category of the timing of viral clearance was the same, the respective rates for SVR attainment according to the mean doses of both Peg-IFN and ribavirin were similar. Furthermore, multivariate analysis by the Mantel–Haenszel chi-square test showed that neither the mean dose of Peg-IFN (P = 0.795) nor ribavirin (P = 0.649) affected SVR rates after stratification of the timing of viral clearance. Among the patients with RVR, SVR rates were as high as 88–100% regardless of Peg-IFN alpha-2b medication, and the least medicated group (<0.9 μg/kg/week, the mean dose with SD was 0.77 ± 0.10 μg/kg/week, 0.50–0.89) showed 100% of SVR rate (19/19). Similarly, SVR rates were as high as 91–94% regardless of ribavirin medication among the patients with RVR, and 17 of 18 patients (94%) in the least medicated group (<8 mg/kg/day, the mean dose with SD was 6.9 ± 0.90 mg/kg/day, 5.0–7.9)

Table 4 Factors associated with RVR among patients who completed the treatment – univariate analysis

Factor	RVR (n = 133)	Non-RVR (n = 80)	P-value
Age (years)*	51.9 ± 12.3	56.3 ± 11.3	0.010
Sex (male/female)	60/73	32/48	0.279
Body weight (kg)*	60.2 ± 11.6	58.6 ± 11.9	0.276
Body mass index (kg/m^2)*	22.9 ± 3.2	22.6 ± 3.1	0.369
HCV RNA (KIU/mL)†	1050	1800	0.001
Past IFN therapy (naive/experienced)‡	97/34	50/29	0.068
Fibrosis (F 0–2/3–4)§	86/8	50/7	0.315
Activity (A 0–1/2–3)§	51/43	31/26	1.000
White blood cells (per mm^3)*	5300 ± 1760	4850 ± 1400	0.205
Neutrophils (per mm^3)*	2740 ± 1290	2530 ± 1090	0.340
Red blood cells (×10^4/mm^3)*	440 ± 45	432 ± 49	0.628
Haemoglobin (g/dL)*	13.9 ± 1.4	13.9 ± 1.4	0.975
Platelets (×10^4/mm^3)*	18.9 ± 6.1	17.5 ± 6.1	0.170
ALT (IU/L)*	87 ± 93	69 ± 52	0.630
γ-GTP (U/L)*	57 ± 71	53 ± 53	0.658
Creatinine (mg/dL)*	0.7 ± 0.1	0.7 ± 0.1	0.203
Mean Peg-IFN dose (μg/kg/week)*	1.31 ± 0.27	1.31 ± 0.29	0.259
Mean ribavirin dose (mg/kg/day)*	10.1 ± 1.8	10.3 ± 2.1	0.637

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; CI, confidence interval. *Values expressed as mean ± SD, †values expressed as median, ‡interferon treatment history was not known for three patients, §data for 62 patients are missing.

Table 5 Factors associated with RVR among patients who completed the treatment – multivariate analysis

Factor	Category	Odds ratio	95% CI	P-value
Age (years)	By 10	0.648	0.494–0.850	0.002
HCV RNA (KIU/mL)	By 100 KIU/mL	0.964	0.944–0.984	<0.0001

HCV, hepatitis C virus; CI, confidence interval.

achieved SVR. In addition, we examined the drug impact on SVR in the patients with the least medication of both drugs (<0.9 μg/kg/week of Peg-IFN and <8 mg/kg/day of ribavirin). Nine patients were categorized into this group and six of these patients achieved SVR (67%); patients with RVR had a significantly higher SVR rate (100%, 5/5) than patients without RVR (25%, 1/4; P = 0.048). Thus, SVR attainment was dependent on time, not on drug dose.

DISCUSSION

In the present study, we found that having RVR and high platelet counts were statistically associated with reaching SVR according to multivariate analysis. The timing of viral clearance was closely related to the treatment effect in patients with genotype 2, similar to the case for those with genotype 1. Ninety-two per cent of SVR was observed for patients with RVR and, conversely, 96% of the patients with HCV RNA positivity at week 8 showed non-SVR. The predictability of SVR based on EVR, defined as a decline of at least 2-log from the baseline of the HCV RNA level at week 12, has been assessed, and genotype 1 patients who have failed to reach EVR are recommended to discontinue the treatment after 12 weeks, because the likelihood of SVR is 0–3% in the absence of EVR [5,13]. On the basis of our examination of patients with genotype 2, not EVR, but 8-week monitoring of the HCV RNA level can be used.

As a significant factor for SVR, not liver fibrosis, but the platelet count was selected. Everson *et al.* [14] reported that patients with low platelet counts (≤12.5 × 10^4/mm^3) achieved lower SVR rates than patients with normal platelet counts (>12.5 × 10^4/mm^3) even in the case of patients with the same category of liver fibrosis treated by Peg-IFN plus ribavirin combination therapy. Thus, independent of liver fibrosis, thrombocytopenia itself seems to participate in treatment failure, although the mechanism remains unknown.

Our study also demonstrated that younger age (OR 0.648, 95% CI 0.494–0.850, P = 0.002) and lower HCV RNA level (OR 0.964, 95% CI 0.944–0.984, P < 0.0001) were statistically associated with reaching an RVR. Zeuzem *et al.* [7] previously reported that pretreatment viral load was not

Table 6 SVR rates according to Peg-IFN alpha-2b and ribavirin exposure and the timing of viral clearance among patients with virological response during the treatment

Timing of viral clearance (week)	Peg-IFN dose (μg/kg/week)				Ribavirin dose (mg/kg/day)				Total
	<0.9	0.9–1.2	1.2–1.5	1.5≤	<8	8–10	10–12	12≤	
1–4	100% (19/19)	91% (10/11)	92% (65/71)	88% (28/32)	94% (17/18)	92% (33/36)	91% (51/56)	91% (20/22)	92% (122/133)
5–8	63% (5/8)	33% (1/3)	64% (19/30)	71% (12/17)	58% (7/12)	54% (7/13)	74% (17/23)	60% (6/10)	64% (37/58)
9–24	–	0% (0/1)	17% (1/6)	–	–	0% (0/1)	0% (0/4)	50% (1/2)	14% (1/7)
Total	89% (24/27)	73% (11/15)	79% (85/107)	82% (40/49)	80% (24/30)	80% (40/50)	82% (68/83)	79% (27/34)	81% (160/198)

*P = 0.795 for comparison of the four Peg-IFN groups after stratification of the timing of viral clearance. **P = 0.649 for comparison of the four ribavirin groups after stratification of the timing of viral clearance.

associated with reaching RVR in genotype 2 patients. In contrast, Dalgard *et al.* [15] reported that independent predictors of RVR in genotype 2 or 3 patients were male gender, younger age (≤40 years) and low viral load (≤400/KIU/mL). The influence of viral load on reaching RVR remains controversial in the Peg-IFN and ribavirin combination therapy in genotype 2 patients, but patients with lower viral load seem favoured to reach HCV RNA levels below the detection limit, that is, to attain RVR, if the virological response is the same.

Recently, because of substantial adverse effects and costs associated with this therapy, studies have been carried out to determine the possibility of further reducing the total amount of drug medication without compromising antiviral efficacy in HCV genotype 2 and 3 patients. There seem to be two ways to achieve. One is by shortening the treatment duration, and the other is by decreasing the doses of the treatment drugs. With respect to the former, several studies on genotype 2 patients have been reported. At first, some studies of small numbers of subjects demonstrated that cumulatively analysed genotype 2 and 3 patients had high SVR rates up to 12 to 16 weeks of therapy (82–94%), similar to patients subjected to 24-week therapy (76–95%) [16–19]. However, further prospective investigation of large numbers of subjects revealed that shortening the treatment duration was associated with an increase in the rate of relapse and that significantly higher relapse rates led to lower SVR rates (71–81.1%), even among those with RVR [15,20,21]. The latest study by Mangia *et al.* [22] showed that shortened therapy after RVR was acceptable only for patients who had no signs of advanced liver fibrosis and low BMI. Considering the results of these trials, shortened therapy is regarded as optional treatment for selected patients displaying favourable baseline characteristics. Therefore, shortening treatment duration from 24 weeks should not be generally recommended for patients who are infected genotype 2 or 3 and can tolerate 24-week Peg-IFN and ribavirin combination therapy.

Another attempt to improve the treatment tolerability for genotype 2 or 3 patients has focused on dose reduction of treatment drugs. Weiland *et al.* [23] examined low-dose Peg-IFN alpha-2a (135 μg weekly) with a weight-based standard-dose of ribavirin (11 mg/kg daily) for genotype 2 and 3 patients. They demonstrated that SVR rates of 86% were achieved, which is equal to those in previous representative randomized controlled studies of standard dose Peg-IFN therapy (76–84%) [4–6]. In contrast, Ferenci *et al.* [24] examined the efficacy of standard-dose Peg-IFN alpha-2a (180 μg weekly) with low-dose ribavirin (400 mg daily) in comparison with standard-dose Peg-IFN alpha-2a (180 μg weekly) and ribavirin (800 mg daily) for genotype 2 and 3 patients, and demonstrated that there was no difference between the two treatment groups with respect to SVR rates (64% with 400 mg/day compared with 69% with 800 mg/day) and relapse rates (20% with 400 mg/day compared

with 17% with 800 mg/day). These studies showed that either drug dose can be reduced for genotype 2 and 3 patients without compromising antiviral efficacy. In the present study, neither Peg-IFN nor ribavirin drug exposure participated in reaching RVR and SVR. In particular, more than 90% of patients having RVR achieved SVR regardless of the drug exposure level, as long as the mean Peg-IFN dose was over 0.5 μg/kg/week and ribavirin was over 5.0 mg/kg/day. The results of our study suggested that genotype 2 patients may receive reduced levels of both drug doses on the condition that they can complete the full 24-week course of combination therapy. Randomized, prospective trials that reduced both Peg-IFN and ribavirin should be conducted for CHC patients to clarify this.

In the present study, while the treatment outcome was independent of the individual ribavirin exposure in patients who had completed the 24-week treatment, the most common reason to withdraw the treatment was decreased haemoglobin because of ribavirin medication. Based on the results of randomized controlled trials [6], using a ribavirin dose of 800 mg/day is recommended for genotype 2/3 patients [1–3]. However, several studies have shown that some patients cannot tolerate even this suboptimal ribavirin dose. This is a serious problem for patients with the risk of anaemia, especially elderly patients. The ageing of patients is progressing around the world, requiring improvement in treatment tolerability. Recently, Andriulli *et al.* [25] examined the effect of ribavirin in a 12-week course of therapy on CHC genotype 2 patients with RVR in two groups, one continuing with ribavirin and the other receiving Peg-IFN alpha-2a alone after week 6. The relapse rates were higher (46% *vs* 17%; $P < 0.001$) and overall SVR rates were lower (54 *vs* 82%; $P < 0.001$) in patients who stopped receiving ribavirin at week 6. Thus, ribavirin medication throughout the treatment period is necessary to raise the SVR rate even in genotype 2 or 3 patients with RVR. In the present study, the ribavirin dose could be reduced without loss of efficacy for genotype 2 patients, as long as the patients were treated for 24 weeks. Therefore, in the patients with the risk of anaemia, it would be better to reduce the dose of ribavirin before anaemia arises rather than being forced to discontinue the combination therapy because of anaemia caused by ribavirin medication. We previously reported that in CHC patients treated by IFN or Peg-IFN in ribavirin combination therapy, a decline of haemoglobin concentration by 2 g/dL at the end of 2 weeks from the start of the treatment can be used to identify patients likely to develop severe anaemia [26,27]. This kind of predictive factor for the progression to severe anaemia can be of much help in reducing ribavirin with appropriate timing.

Our study has some limitations. First, it is a retrospective study, and we could not obtain complete information for all patients. However, this is the first study of Peg-IFN and ribavirin combination therapy in which the drug dose of Peg-IFN and ribavirin taken by each patient was assessed independently for HCV genotype 2 patients. Our results can be taken as an evidence offering suggestions for the treatment of CHC genotype 2 patients. Second, this cohort included patients with different histories of past IFN treatment. Patients who had failed to recover with previous IFN-based treatment were likely to experience treatment failure again [28]. Therefore, we examined the predictors of treatment response separately according to treatment history, and confirmed that in both naïve and treatment-experienced patients, the mean dose of Peg-IFN and ribavirin showed no correlation with SVR or RVR in both groups.

In conclusion, our study demonstrates that RVR is an important treatment predictor and more than 90% of patients having RVR achieve SVR with combination therapy of Peg-IFN and ribavirin for genotype 2 infected CHC patients regardless of the drug exposure. Further prospective, randomized studies are necessary to assess whether the standard or a reduced dose of each drug can produce equivalent outcomes.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Absence of invariant natural killer T cells deteriorates liver inflammation and fibrosis in mice fed high-fat diet

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Abstract
Background Invariant natural killer T (iNKT) cells have been suggested to play critical roles in a wide range of immune responses by acting in a proinflammatory or anti-inflammatory manner. Nonalcoholic steatohepatitis (NASH) is a chronic liver disease progressing to advanced cirrhosis and hepatocellular carcinoma. Despite the abundance of iNKT cells in the liver, their role in the pathogenesis of NASH remains obscure. Here, we investigated their role in the development of diet-induced steatosis/steatohepatitis.
Methods We used BALB/c wild-type mice and Jα18-deficient (KO) mice lacking iNKT cells fed either a normal diet or a high-fat diet (HFD). The liver and blood were collected from these mice to examine liver inflammation, steatosis, and fibrosis at the indicated time points.
Results KO mice fed the HFD, compared with control mice fed the HFD, exhibited a clearly higher serum alanine aminotransferase level and a greater number of hepatic inflammatory foci, although there was no significant difference in hepatic lipid retention between these groups of mice. The HFD enhanced hepatic messenger RNA expression of inflammatory cytokines and chemokines in KO but not in control mice. The HFD also increased the proportion of hepatic CD4 T cells and CD8 T cells that composed hepatic inflammatory foci in KO mice, but not in the controls. Prolonged feeding with the HFD augmented liver fibrosis in KO but not in control mice.
Conclusions These findings indicate that iNKT cells play a protective role against liver inflammation progressing to fibrosis, but not against steatosis, enhanced by dietary excess fat, suggesting a key role of these cells in NASH pathogenesis.

Keywords iNKT cells · Nonalcoholic fatty liver disease · Nonalcoholic steatohepatitis · Cytokine · Chemokine

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
iNKT	Invariant natural killer T
NK	Natural killer
TCR	T cell receptor
Th	T helper
IFN	Interferon
IL	Interleukin
WT	Wild type
ND	Normal diet
HFD	High-fat diet
KO	Jα18-deficient
ALT	Alanine aminotransferase
RT-PCR	Reverse transcription polymerase chain reaction
H&E	Hematoxylin–eosin
SEM	Standard error of the mean
TNF	Tumor necrosis factor
CCL	Chemokine (C–C motif) ligand
CXCL	Chemokine (C–X–C motif) ligand

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders ranging from nonalcoholic steatosis to nonalcoholic steatohepatitis (NASH), which can develop to progressive disease including advanced liver fibrosis and hepatocellular carcinoma [1, 2]. Prolonged overnutrition causes accumulation of free fatty acid and triglycerides within the liver, which is referred to as steatosis. Simple steatosis leads to a predisposition for steatohepatitis, which exhibits inflammatory cell accumulation and fibrosis in the liver in addition to the steatosis [1, 2]. To transform from steatosis to steatohepatitis, several key biological responses such as oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and abnormal cytokine properties have been reported to be required [1–4]. However, the immunological aspect, in particular, that is involved in the development of steatosis/steatohepatitis remains to be fully elucidated.

Invariant natural killer T (iNKT) cells are characterized by the expression of surface markers of natural killer (NK) cells together with a single invariant T cell receptor (TCR) encoded by Vα14-Jα18 in mice and Vα24-Jα18 in humans [5]. These cells are included within the population of T cells expressing NK cell markers, also known as NKT cells [5, 6]. iNKT cells recognize glycolipid antigens presented in association with the major histocompatibility complex class Ib molecule CD1d [5], which is expressed on a variety of cells including dendritic cells, B cells, and stellate cells, as well as hepatocytes in the liver [5, 7, 8]. Following the recognition of antigens via TCR, iNKT cells have the ability to produce the T-helper (Th) 1 cytokine, interferon (IFN)-γ, and the Th2 cytokines, interleukin (IL)-4, -5, and -13, modulating subsequent immune responses [5, 6, 9]. These cells have been shown to play a proinflammatory role in some immune responses and an anti-inflammatory role in other immune responses [5, 6, 9]. iNKT cells most frequently reside in the liver in mice [10, 11]. Although humans appear to have proportionally fewer iNKT cells than mice, human iNKT cells also preferentially reside in the liver [12, 13]. Several lines of evidence indicate that the number of NKT cells is dysregulated in the development of NAFLD. Hepatic iNKT cells or NK1.1+ CD3+/TCRβ+ NKT cells, for instance, have been reported to decrease with the development of steatosis in wild-type (WT) as well as leptin-deficient ob/ob mice [14–17]. A reduced level of peripheral Vα24+ NKT cells has been associated with human NAFLD [18]. On the other hand, CD56+ CD3+ NKT cells have been recently reported to be increased in the livers of patients with NAFLD [19]. Also, the adoptive transfer of NK1.1+ CD3+ NKT cells has been shown to alleviate hepatic steatosis in ob/ob mice [20]. However, the precise role of NKT cells in the pathogenesis of NAFLD has not been investigated in the presence of a deficiency of these cells.

In the present study, we used iNKT cell-deficient as well as WT mice fed either a normal diet (ND) or a high-fat diet (HFD), and examined the role of these cells in the development of HFD-induced steatosis/steatohepatitis. We found that the lack of iNKT cells, together with the HFD, led to liver inflammation, which was characterized by the enhanced gene expression of inflammatory cytokines and chemokines and by T cell accumulation. We also found that prolonged liver inflammation in the absence of iNKT cells developed to liver fibrosis which was strongly enhanced by the HFD. This study delineated an immunoregulatory function of iNKT cells and their key role against liver inflammation progressing to fibrosis exacerbated by an HFD, which might represent a clinical aspect of human progressive NAFLD.

Materials and methods

Animals and animal care

Specific pathogen-free BALB/c WT mice were purchased from CLEA Japan (Tokyo, Japan) as needed. Breeding pairs of BALB/c Jα18-deficient (KO) mice [21, 22] were provided by Drs. Masaru Taniguchi and Ken-ichiro Seino (RIKEN, Yokohama, Japan). The KO mice were confirmed to have no iNKT cells by the use of mouse-CD1d tetramers loaded with α-galactosylceramide in the flow cytometry procedure described below (data not shown). These mice were kept in isolation facilities at the Institute of Experimental Animal Science, Osaka University. They were housed in groups of five in filter cages and were maintained in a temperature-controlled, specific-pathogen-free room on 12-h light and dark cycles with ad libitum access to water and diet as indicated.

Experimental protocol

Male mice used in the experiments were fed an irradiated HFD consisting of 56.7% of the calories from fat (HFD32; CLEA Japan) or an irradiated ND consisting of 14% of the calories from fat (CRF-1; Oriental Yeast, Osaka, Japan), starting from when the mice were 6–8 weeks old. In preliminary experiments, we monitored the body weight of the WT mice and KO mice fed the ND or HFD every 2 weeks after the initiation of feeding, because a gain of body weight usually parallels the level of hepatic steatosis as well as obesity. We did not observe a gain of body weight of more than 25% until 4 weeks after the initiation of feeding. In mice fed the HFD, the body weight gain reached a plateau around 14–16 weeks after the initiation

of feeding. These observations led us to set the time point for estimating liver steatosis and injury and inflammation or liver fibrosis during the course of feeding at week 5 or week 15, respectively. At the end of the indicated periods, the mice were weighed and anesthetized with pentobarbital sodium, and then their abdomens were opened. Following blood sampling via the inferior caval vein, the portal vein and inferior caval vein were cut to enable blood outflow and then the liver was removed, weighed, and processed for further analyses. All animal experimental protocols were approved by the Institute of Experimental Animal Science, Osaka University. To evaluate the levels of liver injury, serum alanine aminotransferase (ALT) activities were measured as previously described [23]. To determine the levels of steatosis, total lipids were extracted from the liver and then triglyceride content was measured as previously described [24].

Flow cytometric analysis

Liver mononuclear cell populations were prepared as previously described [11, 23]. Cell surface staining of the prepared cells was performed as described [11, 23], using the following antibodies or tetramers: fluorescein isothiocyanate-conjugated anti-CD49b (DX5), phycoerythrin-conjugated anti-CD4 (H129.19), peridinin chlorophyll protein-conjugated anti-CD8α (53-6.7), and allophycocyanin-conjugated anti-TCRβ (H57-597) monoclonal antibody, or fluorescein isothiocyanate-conjugated anti-TCRβ, phycoerythrin-conjugated anti-CD4, peridinin chlorophyll protein-conjugated anti-CD45R/B220 (RA3-6B2) monoclonal antibody, and allophycocyanin-conjugated mouse-CD1d tetramers loaded with α-galactosylceramide. All antibodies were purchased from BD Biosciences (San Jose, CA, USA). Mouse CD1d tetramer was obtained from Proimmune (Oxford, UK) and the loading with α-galactosylceramide was performed following the manufacturer's protocol. The stained cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA), and the data were processed using the CELLQuest program (Becton Dickinson). iNKT cells were detected on electronically gated CD45R/B220- TCRβ+ CD1d-tetramer-reactive cells.

RNA isolation and analysis

Total RNA was isolated from frozen liver tissues by using an RNeasy kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Complementary DNA was synthesized from isolated RNA using SuperScript III and random hexamer (Invitrogen, Carlsbad, CA, USA). Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) normalized to beta-actin.

Histological evaluation

The removed liver was partly fixed in 10% formalin for staining with hematoxylin–eosin (H&E), Sirius-Red, or Oil-red-O, or it was immediately embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen for immunohistochemical staining. Sirius-Red staining was performed to assess liver fibrosis, which was quantified by the extent of the area, using image-analysis software, WinROOF (Mitani, Fukui, Japan). Intracellular lipid was stained with Oil-red-O. To evaluate the infiltration of CD4+ cells or CD8+ cells into the liver, acetone-fixed fresh-frozen tissue sections were immunostained with anti-mouse CD4 (H129.19) or anti-mouse CD8α (53-6.7) monoclonal antibody, respectively, using a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol. The sections were developed with diaminobenzidine (DAB) substrate (Vector Laboratories) and then counterstained with hematoxylin. Antibody against CD4 or CD8 was purchased from BD Biosciences.

Statistical analysis

The statistical significance of differences between two groups was determined by applying the Mann–Whitney *U*-test. Statistical significance was defined as $P < 0.05$. All data are shown as mean ± standard error of the mean (SEM).

Results

Lipid accumulation in the liver induced by the HFD was independent of the presence or absence of iNKT cells

To investigate the role of iNKT cells in the development of diet-induced steatosis/steatohepatitis, we fed the ND or HFD to WT and KO mice for 5 weeks. The HFD increased the body weight by around 30% at week 5 in both WT and KO mice, while the ND increased it by around 14% (HFD-fed WT mice 31.6 ± 2.4%, HFD-fed KO mice 29.7 ± 5.6%, ND-fed WT mice 15.5 ± 0.6%, ND-fed KO mice 13.5 ± 1.1%; $n = 5$). The weight gains with the HFD or ND were not significantly different between WT and KO mice. Evaluation of the liver weight at week 5 showed that the HFD-fed WT or KO mice possessed significantly heavier livers than the ND-fed WT or KO mice, respectively, without any significant differences between the WT and KO mice (HFD-fed WT mice 1.95 ± 0.06 g, HFD-fed

KO mice 1.89 ± 0.07 g, ND-fed WT mice 1.52 ± 0.04 g, ND-fed KO mice 1.50 ± 0.06 g; $n = 5$).

We next performed Oil-red-O staining of liver sections from the mice to examine whether the absence of iNKT cells would affect the HFD-induced lipid accumulation in the liver. The staining showed that the HFD, compared with the ND, induced marked lipid retention in hepatocytes in both WT and KO mice (Fig. 1a). Evaluation of the liver triglyceride level demonstrated that the HFD, compared with the ND, clearly induced triglyceride accumulation in the livers of both WT and KO mice, without a significant difference between these groups of mice (Fig. 1b).

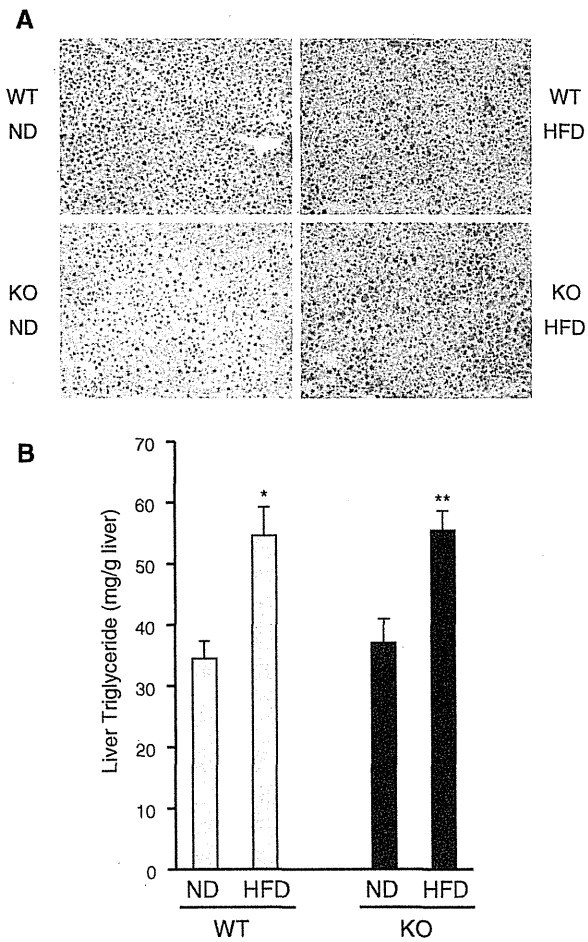

Fig. 1 Lipid accumulation in the liver induced by high-fat diet (*HFD*). Livers were obtained from BALB/c wild-type (*WT*) and BALB/c Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or an HFD for 5 weeks. **a** Lipid accumulation in liver sections was visualized by Oil-red-O staining. Representative images are shown (×200). **b** Hepatic triglyceride levels were quantified. Data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments. $^*P < 0.05$ versus WT fed ND. $^{**}P < 0.05$ versus KO fed ND

Collectively, these results suggested that the absence of iNKT cells did not affect the level of HFD-induced steatosis.

HFD augmented liver injury and inflammation in the absence of iNKT cells

To examine the levels of liver injury, we measured ALT activity in serum from WT and KO mice fed the ND or HFD at week 5 after the start of being fed the diets. The serum ALT level in the HFD-fed WT mice (35.8 ± 1.98 IU/l) was significantly higher than that in the ND-fed WT mice (25.2 ± 0.66 IU/l) (Fig. 2a). The serum ALT level in the HFD-fed KO mice (174.8 ± 61.2 IU/l) was also significantly higher than that in the ND-fed KO mice (36.4 ± 7.48 IU/l). It was also higher than that in the HFD-fed KO mice at week 2 (83.3 ± 16.5 IU/l). Of note is the finding that the magnitude of the increase in ALT level at week 5 was clearly much higher in KO (4.9-fold) than in

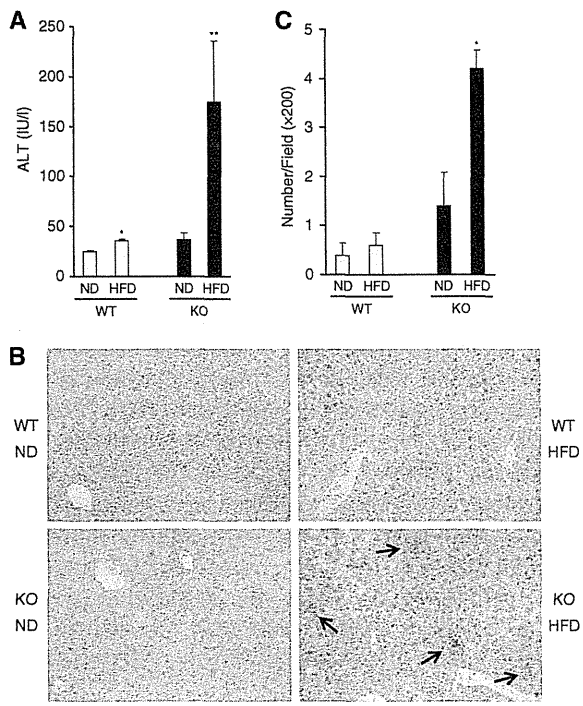

Fig. 2 Liver injury and inflammation exacerbated by HFD in the absence of invariant natural killer T (*iNKT*) cells. Serum and livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. **a** Serum alanine aminotransferase (*ALT*) levels were measured. $^*P < 0.05$ versus WT fed ND. $^{**}P < 0.05$ versus KO fed ND. **b** Liver tissues were stained with hematoxylin–eosin. Representative images are shown (×200). *Arrows* indicate the inflammatory foci. **c** The numbers of the foci were counted in five different fields per section. $^*P < 0.05$ versus KO fed ND. All data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments