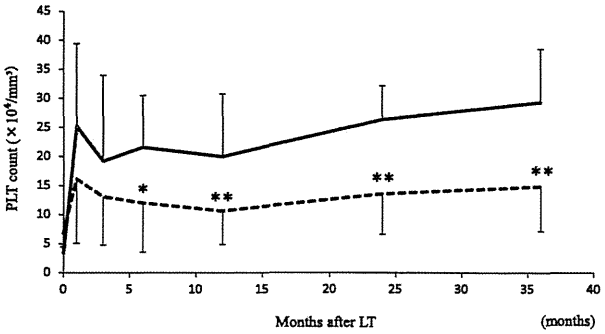

Fig 2. The kinetics of platelet count in each group with or without simultaneous splenectomy according to the criterion. The solid line indicates the kinetics of mean platelet count of the recipients who required receiving simultaneous splenectomy (n = 6). The dotted line indicates the kinetics of mean platelet count of the recipients who did not require receiving simultaneous splenectomy (n = 30) (mean ± standard deviation, $^{*}P < .05$, $^{**}P < .01$).

enough level for initiating IFN therapy at the early time of after LT, and subsequently maintained enough level for continuing IFN therapy. Furthermore, the kinetics of the PLT count after LT, in the individual recipient who did not require simultaneous splenectomy, is shown in Fig 3. In 16 (66.7%) recipients from this group, the PLT count increased to >100,000/mm^3 1 month after LT. The average PLT counts pre-LT and 1 month after LT were 7.0 ± 2.2/mm^3 and 11.7 ± 5.2/mm^3, respectively. Twenty-one (87.5%) recipients could start IFN therapy and the other 3 recipients could not because of liver failure, purulent spondylitis, or spontaneous remission.

DISCUSSION

In LT recipients, splenectomy is mainly performed for the purpose of improving thrombocytopenia due to hypersplenism, controlling portal vein pressure in small-for-size syndrome, and/or immunotherapy for ABO incompatibility [11–16]. Splenectomy itself potentially has disadvantages associated with multiple complications, such as portal vein thrombosis, pancreatic leaks, and infection [15,17–20]. Among them, the most serious complication is considered to be infections such as overwhelming post-splenectomy infection (OPSI), especially in immunosuppressed recipients after LT [17]. Immunizing recipients before splenectomy to decrease the risk of OPSI is recommended [21], although the response rate of vaccinations is reportedly only 40% to 80% in LT recipients [22,23]. Therefore, we established the criterion for simultaneous splenectomy to avoid unnecessary splenectomy in the LT recipients with HCV infection and evaluated the validity of this criterion in this study.

In the present study, the PLT counts of the recipients who received splenectomy according to the criterion increased immediately, and all of them completed the IFN therapy. However, one of them suffered from a bloodstream infection caused by diplococcus pneumonia that was considered an OPSI. On the other hand, the recipients who did not require simultaneous splenectomy according to the criterion also reached the appropriate level of PLT count and achieved reasonable outcomes. However, 8.3% of these patients required subsequent metachronous splenectomy to continue IFN therapy.

To improve the accuracy of our criterion, we investigated the functional variant in the inosine triphosphatase (*ITPA*) gene of the recipients. The functional single-nucleotide

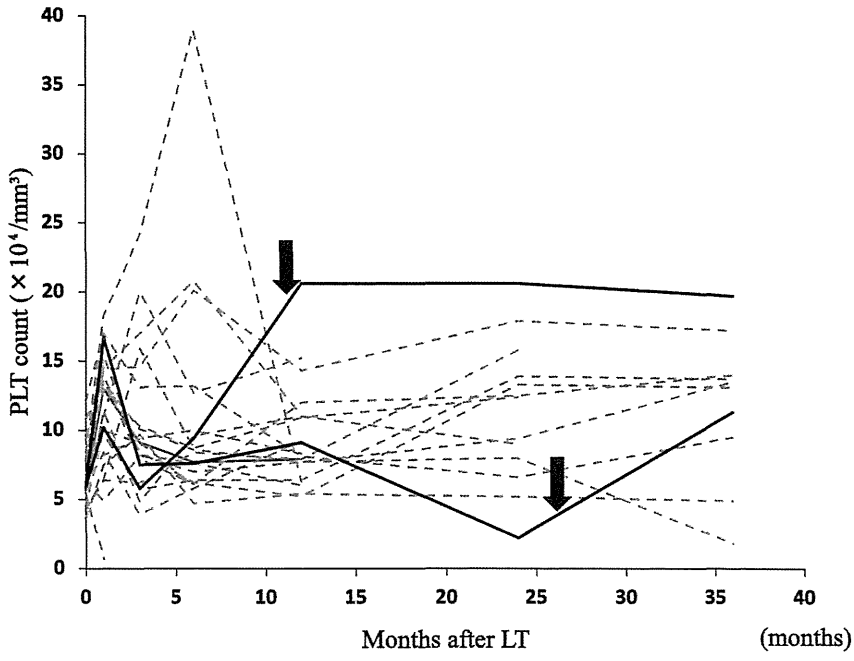

Fig 3. The kinetics of platelet count in the recipients who did not require simultaneous splenectomy according to the criterion. The black solid and gray dotted lines indicate the kinetics of platelet (PLT) count after liver transplantation in the recipients who did not require receiving simultaneous splenectomy. The black solid lines indicate the PLT count in the recipients who required metachronous splenectomy for continuing interferon therapy. The black arrows indicate the timing of metachronous splenectomy.

polymorphism (SNP) of the *ITPA* gene is associated with RBV-induced anemia and IFN-induced thrombocytopenia in Japanese genetic populations [24]. Severe anemia induced by RBV, which is mainly found in patients with *ITPA-CC* (major variant), was inversely correlated with thrombocytopenia. The functional variant in the *ITPA* gene was identified in the 13 recipients who belonged to the group that did not require simultaneous splenectomy. Twelve of the 13 recipients had *ITPA-CC* variants that are protective against IFN-induced thrombocytopenia, although 2 recipients who required metachronous splenectomy for continuing IFN therapy had the CC variants. However, the small number of recipients investigated here did not allow us to analyze whether this SNP could predict the outcome.

In conclusion, our criterion detected the outcome of PLT count after LT in recipients with HCV and achieved better result of SVR after IFN therapy. However, further factors may need to be identified to improve the prediction of thrombocytopenia in HCV recipients after LT.

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ELSEVIER

Potential Benefit of Mixed Lymphocyte Reaction Assay-based Immune Monitoring After Living Donor Liver Transplantation for Recipients With Autoimmune Hepatitis

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ABSTRACT

Background. Recipients with autoimmune hepatitis (AIH) have a higher incidence of both rejection and recurrence after liver transplantation (LT) when compared with cholestatic liver diseases such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). This is due to the lack of an immune monitoring system, making it difficult to control immunosuppressant agents. In this study, we examine the benefit of the carboxyfluorescein diacetate succinimidyl ester–mixed lymphocyte reaction (CFSE-MLR) monitoring system for evaluating the immune status in recipients with AIH and PBC/PCS after LT.

Method. Recipients who underwent LT (9 AIH and 11 PBC/PSC) from 2002 to 2013 at Hiroshima University were enrolled in this study. The correlation between the result of CFSE-MLR and the outcome, bacteremia, rejection, and/or recurrence was examined.

Result. The cumulative survival rates for 5 years after LT revealed preferable outcomes for both groups (AIH 85.7%, PBC/PCS 80%). None of the recipients in the AIH group developed bacteremia during 90 days after LT, whereas three recipients from the PBC/PCS group (27%) developed bacteremia. The recurrence rate (AIH 33%, PBC/PSC 27%) was the same as the reported data; however, there was a lower incidence of acute rejection rate in our institution (AIH 11%, PBC/PSC 27%). In the CFSE-MLR assay, the stimulation index of $CD4^+$ T cells in the anti-self reaction was increased in recurrent cases, whereas no elevation of anti-donor reaction was observed in either $CD4^+$ or $CD8^+$ T cells.

Conclusion. Optimization of the immunosuppressant agents based on the CSFE-MLR assay after LT achieved a preferable outcome in recipients with both AIH and PBC/PCS. Therefore, CFSE-MLR assay might be a useful tool for predicting the recurrence of autoimmune liver diseases by monitoring anti-self reactivity of $CD4^+$ T cells.

AUTOIMMUNE liver diseases, including autoimmune hepatitis (AIH) and cholestatic liver diseases such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), may require liver transplantation (LT) in patients who are refractory to immunosuppressive therapy and/or in whom end-stage liver disease develops [1]. AIH is reported to be categorized in a high-risk group for rejection and recurrence after LT and to have a comparatively poorer prognosis than PBC and PSC [2–4]. Recipients with AIH often require a higher dose and a more prolonged course of corticosteroids after LT; this may predispose them to the

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various side effects of steroids, including a risk of infection. However, early withdrawal of steroids may increase the risk of recurrence as well as the risk of acute rejection. The pathogenesis of AIH is unknown and AIH is believed to be a disease of disordered immune regulation. Sensitized T cells in AIH recipients after LT release proinflammatory cytokines that cause hepatocellular damage and graft dysfunction and occur as a result of recurrent autoimmune disease [5]. Additionally, the loss of self-tolerance in AIH recipients may be an important component of alloimmune responses in the liver allograft.

Therefore, recipients who have autoimmune liver diseases must perform strict and careful immune monitoring after LT to prevent both rejection and recurrence [6,7]. In our institution, we have been monitoring immunosuppressive status in each recipient who underwent LT for any disease and adjusted the optimum immunosuppressant dosage individually by using the carboxyfluorescein diacetate succinimidyl ester–mixed lymphocyte reaction (CFSE-MLR) assay [8]. In this study, we compared immune states and outcomes between AIH and cholestatic liver diseases (PBC and PSC) after LT and evaluated the benefit of CFSE-MLR as a tool for immune monitoring with AIH recipients.

PATIENTS AND METHODS

Patients

From January 2002 to July 2013, a total of 193 patients who received living donor LT at the Hiroshima University were enrolled in this study. Of these, 9 recipients (4.7%) were preoperatively diagnosed with AIH. Eight (4.1%) and 3 (1.6%) recipients were diagnosed with PBC and PSC, respectively. The characteristic data of recipients, such as pretransplantation AIH score, outcome, bacteremia, rejection, and/or recurrence in that AIH and PBC/PSC groups are listed in Table 1.

Immune Monitoring Based on a CFSE-MLR Assay

For CFSE-MLR assay, peripheral blood mononuclear cells (PBMCs) from the recipient, donors, and third-party healthy volunteers were used as stimulators and recipient PBMCs were labeled with CFSE and used as responders. After 5 days of culture, the responder cells were harvested and analyzed by performing flow cytometry. $CD4^+$ and $CD8^+$ T cells were selected through gating and analyzed for intensity of CFSE fluorescence. Stimulation index (SI) could be calculated by using a formula as described previously [9]. Immune states were estimated based on these indices in response to both anti-self and anti-donor versus anti–third party stimulations in CFSE-MLR for predicting the recurrence of autoimmune liver disease. Allograft rejection and immunosuppressant agents were modulated according to the estimated anti-donor reactivity.

Diagnosis of Rejection and Recurrence

We monitored immune status by performing scheduled CFSE-MLR assay after LT. Graft rejections were diagnosed in cases of increased anti-donor alloreactive T cells defined as the number and phenotype of alloreactive precursors in the recipient. When recurrences of AIH, PBC, and PSC and/or acute rejection were suspected by clinical symptoms and the CSFE-MLR assay, graft biopsy

Table 1. Characteristics of AIH and PBC/PSC Recipients

No	Age/Sex	ANA	IgG	HLA DR4	IAIHG AIH score	MELD score	Acute rejection	Recurrence of AIH	Bacteremia in 90 d	Prognosis	Cause of death
AIH											
1	27/M	x320	1900	–	10	36	–	10 m after LT	–	Alive	
2	48/F	x320	3200	DR4	17	22	–	68 m after LT	–	Alive	
3	44/F	x80	2110	DR4	12	24	+	–	–	Alive	
4	50/F	x320	2390	DR4	19	44	–	–	–	Alive	
5	32/F	x2560	5250	DR4	19	21	–	7 m after LT	–	Dead	Liver failure
6	51/F	x1280	2450	DR4	19	14	–	–	–	Alive	
7	67/F	x80	3070	DR4	19	13	–	–	–	Alive	
8	67/F	x1280	3304	–	15	12	–	–	–	Alive	
9	66/F	x80	728	DR4	17	15	–	–	–	Alive	

No	Age/Sex	AMA	Diagnosis	IAIHG AIH score	MELD score	Acute rejection	Recurrence of PBC/PSC	Bacteremia in 90 d	Prognosis	Cause of death
PBC/PSC										
1	51/F	28	PBC	<10	20	–	57 m after LT	–	Alive	
2	52/F	144	PBC	<10	9	–	–	+	Dead	Graft failure Sepsis
3	56/F	35	PBC	10	22	–	–	–	Alive	
4	36/F	138	PBC	<10	16	+	–	–	Alive	
5	46/F	162	PBC/AIH	14	16	–	–	–	Alive	
6	60/F	149	PBC	<10	17	–	31 m after LT	–	Alive	
7	43/F	55	PBC/AIH	17	19	+	–	–	Alive	
8	66/F	100	PBC	12	18	–	–	–	Alive	
9	36/M	N/A	PSC	–	22	+	–	+	Dead	Multiple organ failure
10	36/M	N/A	PSC	–	13	–	23 m after LT	–	Alive	
11	51/F	N/A	PSC	–	16	–	–	+	Alive	

Abbreviations: AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; ANA, antinuclear antibody; HLA, human leukocyto antigen; IAIHG, international autoimmune hepatitis group; MELD score, model for end-stage liver disease score; AMA, antimitochondrial antibody; M, male; F, female; LT, liver transplantation; N/A, not applicable.
IAIHG AIH score (>17: Definite AIH, 12–17: Probable AIH).

specimens were evaluated for each histologic feature. Rejection and/or recurrence was diagnosed based on the histopathologic report.

Definition of Bacteremia

Bacteremia was defined as the isolation of bacteria other than common skin contaminants from a single blood culture obtained within 90 days of LT, or as the presence of clinical symptoms or signs of infection.

Statistical Analysis

Overall survival was calculated by using the Kaplan-Meier method, and SI was compared by using the Student t test. Values of $P < .05$ were considered statistically significant differences.

RESULTS

Recipient Characteristics

The characteristics of recipients in the AIH and PBC/PSC groups are shown in Table 1. Nine of the 193 LT recipients were identified with AIH, including 1 male and 8 female patients. In the PBC/PSC group (11 patients; 2 males and 9 females), 8 (4.1%) and 3 (1.6%) patients were identified with PBC and PSC, respectively. The mean AIH score, calculated by the International Autoimmune Hepatitis Group scoring system, was 16.3 ± 3.4 in AIH recipients and 11.0 ± 3.1 in PBC/PSC recipients. Two of the 8 PBC recipients were diagnosed with PBC/AIH overlap syndrome due to a high AIH score. The mean model for end-stage liver disease (MELD) score in the AIH group was 22.3 ± 11.1, and all the cases except 2 (71%) had HLA-DR4, which is considered a high-risk marker for recurrence. Comparatively, the mean MELD score in the PBC/PSC group was 17.1 ± 3.8, and 5 of the 11 cases (45%) had HLA-DR4. In the CFSE-MLR assay, there was no significant difference in anti-self SI score between the AIH group and the PBC/PSC group at pretransplantation status ($CD4^+$ T cells, 1.5 ± 0.5 vs 1.5 ± 1.0; $CD8^+$ T cells, 1.2 ± 0.2 vs 1.2 ± 0.8).

Recipients' Survival

The median follow-up period was 4.9 years (range, 2 months to 11.1 years). One recipient in the AIH group who developed recurrence of AIH resulting in graft failure died 2.4 years after LT. In the PBC/PSC group, one PBC recipient died 4 months after LT due to portal vein thrombosis, graft failure, and sepsis caused by abdominal infection. One PSC recipient died 2 months after LT because of multiple organ failure caused by sepsis. As shown in Fig 1, the cumulative survival rate for 5 years after LT in the AIH group and PBC/PSC group was 85.7% and 80%, respectively, and showed no significant differences in our institution compared to the reported data [4].

Rejection and Recurrence After Transplantation

In the AIH group, graft rejection was diagnosed at 23 days after LT in one recipient (11%) who was successfully treated with steroid pulse and a monoclonal anti-human T cell antibody therapy without subsequent rejection episodes. In the PBC/PSC group, three recipients (27%) developed acute rejection and were treated with steroid pulse therapy (Table 1). In the AIH group, recurrences after LT were identified in three of nine recipients (33%). Of these recipients, two were successfully treated by increasing corticosteroid doses, whereas the other recipient who was refractory to treatment died of graft failure caused by an acute exacerbation of AIH. Two of the three recurrent recipients, including the recipient who died, had HLA-DR4, which is a high-risk marker of recurrent disease. In the PBC/PCS group, recurrences after LT were identified in 3 of 11 recipients (27%). Of these, 2 PBC recipients developed a recurrence, and 1 PSC recipient who had developed recurrence received a deceased donor LT due to graft failure 4 years after the first living donor LT (Table 1). When we compared the maximum values of SI through the clinical course between recurrent and nonrecurrent cases, the SI of $CD4^+$ T cells against self tended to show a higher response in the recurrent cases than in the nonrecurrent cases (3.1 ± 2.8 and 1.7 ± 0.5, respectively, $P = .20$), although the SI of anti-self reactive $CD8^+$ T cells showed no such differences (Fig 2A). Additionally, no elevation of the anti-donor reaction was observed in either $CD4^+$ or $CD8^+$ T cells in these cases when recurrence developed (Fig 2B).

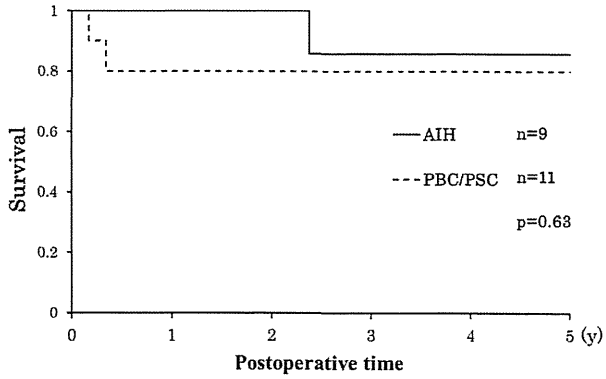

Fig 1. Outcome of AIH and PBC/PSC patients after LT. The cumulative survival rates for 5 years after transplantation in the AIH group and PBC/PSC group were 85.7% and 80%, respectively, with no significant differences.

Bacteremia

No recipients in the AIH group experienced bacteremia during the 90 days after LT. On the other hand, three recipients in the PBC/PCS group (27%) developed bacteremia, and two of these three died because of abdominal infection. Another recipient was diagnosed with acute appendicitis and underwent an appendectomy (Table 1).

DISCUSSION

AIH is reported to be a high-risk factor for rejection and recurrence after LT [2–4]. Reported rates of rejection after

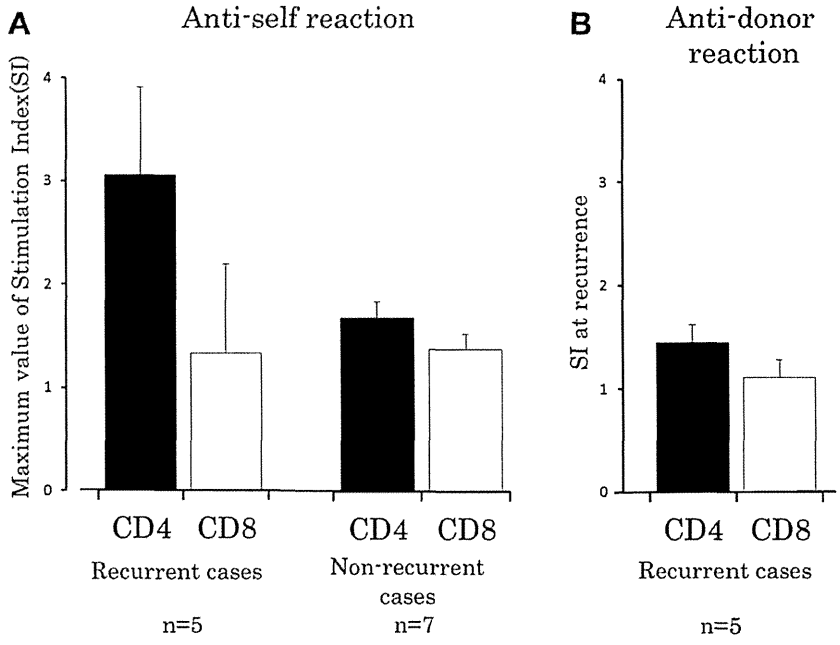

Fig 2. Immune status based on CFSE-MLR monitoring. (**A**) The SI of $CD4^+$ T cells against self tended to be higher in the recurrent cases than in the nonrecurrent cases (3.1 ± 2.8, 1.7 ± 0.5, respectively), although the SI of anti-self-reactive $CD8^+$ T cells showed no such differences (cases more than 1 year after LT). (**B**) No elevation of anti-donor reaction was observed in both $CD4^+$ and $CD8^+$ T cells at the time of recurrence.

LT for AIH, ranging from 50% to 88%, were higher than for other hepatic diseases, whereas our data showed a preferable result with only one recipient of the AIH group (11%) developing an acute rejection [4,10–12]. For infections caused by over-immunosuppression, it is reported that 34% of recipients after LT experienced bacteremia in 90 postoperative days [13], whereas no bacteremia was observed in our cases. The incidence of recurrent AIH in our study is similar to that in previous reports, ranging from 0% to 42% [1,3,4,10]. Most of the recipients in the AIH group had HLA-DR4, which is identified as relevant for susceptibility and/or severity of AIH, suggesting that they are at a high risk for recurrence after LT [6]. In general, clinical and laboratory manifestations can be absent in recipients with recurrent AIH. Deterioration of liver enzymes after LT raises the suspicion of rejection and recurrence, and will usually lead to the need for further investigations. However, the histologic features of disease recurrence may be seen in the presence of normal liver tests. The diagnosis should therefore be based on a combination of serologic, biochemical, and histologic findings; however, serology is of limited help in making the diagnosis of rejection and recurrence. The presence of anti-nuclear antibody and increased immunoglobulin may be preceded by histologic changes, though they are also not helpful markers for determination of AIH recurrence. Therefore, protocol liver biopsy might be of use in the early detection of AIH recurrence. However, liver biopsy is invasive and the features of histopathology at the early phase of AIH recurrence are similar to those of rejection, making it still difficult to distinguish between rejection and recurrence.

In this study, we show that appropriately controlled immune status of the AIH recipients after LT with CFSE-MLR monitoring leads to low incidences of both rejection and infection. We previously reported that optimization of immunosuppressive therapy based on CFSE-MLR assay provides a low incidence of acute rejection and reduces infectious complications after LT [14]. In this study, we evaluated whether recurrence of autoimmune liver disease could be predicted by anti-self reactivity of T cells on CFSE-MLR assay. The SI of $CD4^+$ T cells against self tended to show higher levels in the recurrent cases than in the nonrecurrent cases, although $CD8^+$ T cells showed no such differences. The mechanisms leading to autoimmune liver damage remain unclear, however, it has been reported that liver damage is likely to be orchestrated by $CD4^+$ T cells that recognize a self-antigen peptide. Immunohistochemical studies of interface hepatitis have identified a predominance of $CD4^+$ helper/inducer T cells, and a sizable minority for the $CD8^+$ cytotoxic T cells [15]. To elucidate the clinical significance of these findings, a larger number of these cases is required.

In conclusion, optimization of the immunosuppressant agents based on the CFSE-MLR assay after LT resulted in a low incidence of rejection or bacteremia and achieved a preferable outcome in recipients with AIH. CFSE-MLR assay might become a useful tool for predicting the recurrence of autoimmune liver diseases by monitoring the anti-self-responsibility of $CD4^+$ T cells.

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ELSEVIER

Bile CXC Motif Chemokine 10 Levels Correlate With Anti-donor Cytotoxic T Cell Responses After Liver Transplantation

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ABSTRACT

Background. CXC motif chemokine 10 (CXCL10), known as interferon-γ–induced protein 10, is an inflammatory cytokine secreted by various cells in response to interferon-γ. CXCR3, the receptor of CXCL10, is predominantly expressed on activated T, B, natural killer, and dendritic cells, as well as macrophages. CXCR3 promotes chemotaxis upon binding CXCL10. Serum CXCL10 levels have recently attracted attention as a post-transplantation biomarker for graft rejection. However, the correlation between the degree of T cell response to allostimulation and CXCL10 levels remains unclear. In this study, we investigated the serum and bile CXCL10 levels of patients who underwent living donor liver transplantation (LDLT) and compared them with the T cell responses to allostimulation.

Patients and Methods. Between February 2009 and August 2012, 41 patients underwent LDLT at Hiroshima University Hospital. Serum and bile CXCL10 levels were measured weekly for 4 weeks after surgery, while the T cell responses to allostimulation were evaluated using a mixed lymphocyte reaction with an intracellular carboxyfluorescein diacetate succinimidyl ester–labeling technique that we regularly use to monitor the immune response to anti-donor and anti–third-party stimulation after liver transplantation. The stimulation index (SI) and CD25 expression of the CD4+ and CD8+ T cell subsets in response to allostimulation were then analyzed using flow cytometry.

Results. Serum CXCL10 levels were significantly correlated with the SI values for CD8+ T cells in response to both types of allostimulation. Bile CXCL10 levels were significantly correlated with CD25 expression of CD8+ T cell subsets, especially in response to anti-donor stimulation. Patients with higher bile CXCL10 levels suffered from severe acute cellular rejection that was refractory to steroid pulse.

Conclusion. Measurements of bile CXCL10 levels could predict anti-donor cytotoxic T cell responses in liver transplant recipients.

THE ACCUMULATION of activated immune cells in the allograft is essential to the pathogenesis of tissue injury. CXC motif chemokine 10 (CXCL10), known as interferon-γ (IFN-γ) –induced protein 10 (IP-10), is an inflammatory cytokine secreted by neutrophils, eosinophils, monocytes, and epithelial, endothelial, and interstitial cells in response to IFN-γ [1-4]. CXCR3, the receptor of CXCL10, is predominantly expressed on activated T, B, natural killer, and dendritic cells, as well as macrophages [2,5]. CXCR3 is also reported to promote chemotaxis when

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it binds CXCL10 [6,7]. Recently, CXCL10 levels in sera have been attracting attention as a biomarker for graft rejection after organ transplantation. However, the correlation between the degree of T cell responses to allostimulation and CXCL10 levels remains unclear. In this study, we investigated the serum and bile CXCL10 levels of patients who underwent living donor liver transplantation (LDLT) and compared them with T cell responses to allostimulation.

PATIENTS AND METHODS

Between February 2009 and August 2012, 41 patients underwent LDLT at Hiroshima University Hospital. Serum and bile CXCL10 levels were measured every week for 4 weeks after surgery, and the T cell responses to allostimulation were evaluated by a mixed lymphocyte reaction (MLR) using the intracellular carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeling technique (CFSE-MLR assay) that we regularly use to monitor the immune-state response to anti-donor and anti–third party stimulation after transplantation. The stimulation index (SI) and CD25 expression of the CD4+ and CD8+ T cell subsets in response to allostimulation were analyzed using flow cytometry. Serum and bile CXCL10 levels were assessed using an enzyme-linked immunosorbent assay (Quantikine ELISA Human CXCL10/IP-10; R&D Systems, City of Minneapolis, MN, United States) according to the manufacturer's instructions.

RESULTS

Serum CXCL10 levels were significantly correlated with the SI values of the CD8+ T cells in response to anti-donor and anti–third-party stimulation (Fig 1A), but they did not correlate with the CD25 expression of CD8+ T cell subsets in response to both types of allostimulation (data not

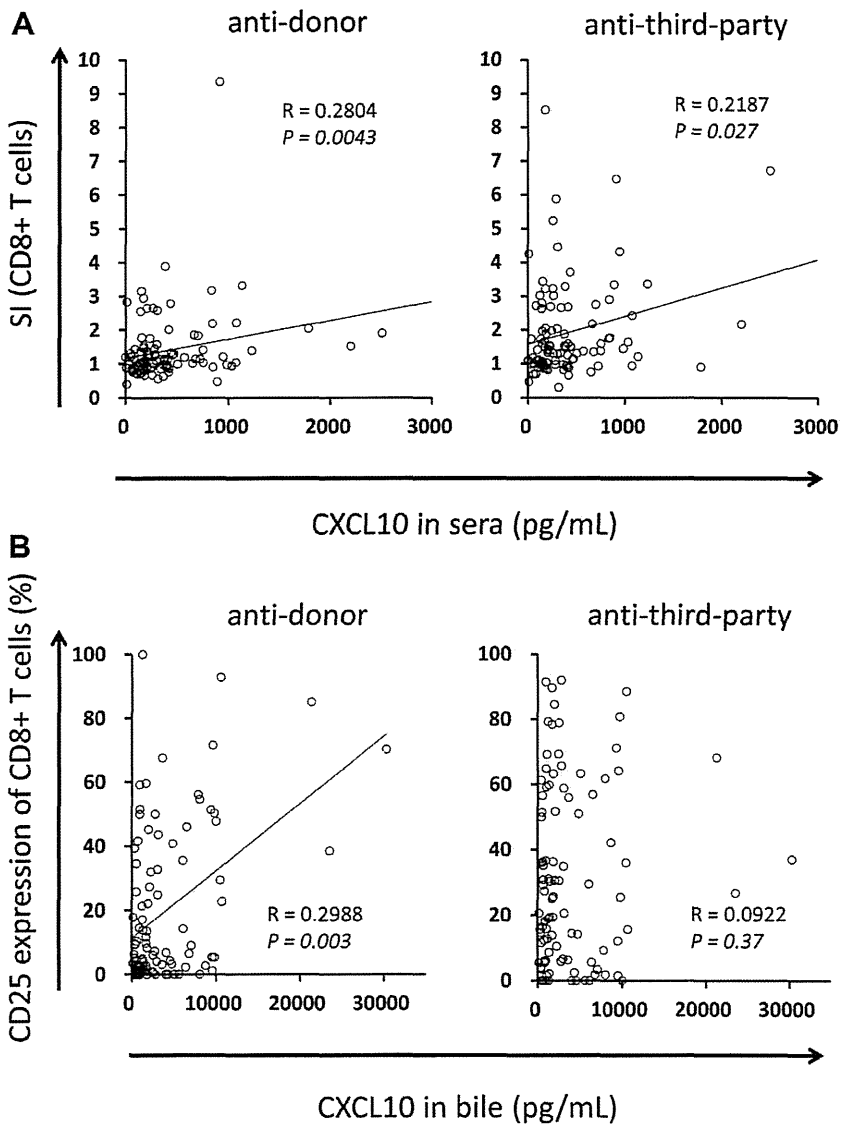

Fig 1. The correlation between serum CXC motif chemokine 10 (CXCL10) levels and the stimulation index values of CD8+ T cells in response to anti-donor or anti–third-party stimulation **(A)** and between CXCL10 levels in the bile and the CD25 expression of CD8+ T cells in response to anti-donor or anti–third-party stimulation **(B)**. The black circles indicate the values of each sample. The lines in the graph indicate the linear approximate curve. The correlations were assessed using the Spearman rank order correlation coefficient, and *P* values < .05 were considered statistically significant.

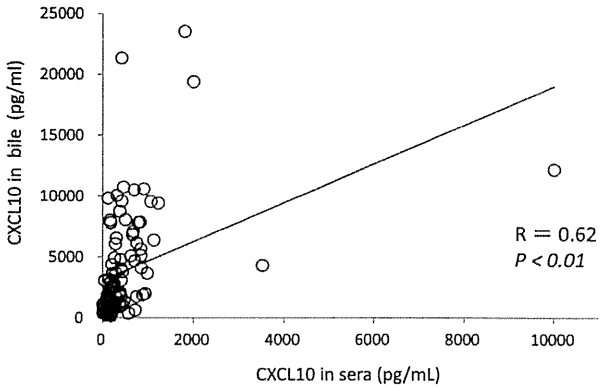

Fig 2. The correlation between serum and bile CXC motif chemokine 10 (CXCL10) levels.

shown). The CXCL10 levels in the bile did not correlate with the SI values of CD8+ T cells in response to both types of allostimulation (data not shown) but were significantly correlated with the CD25 expression of CD8+ T cell subsets specifically in response to anti-donor stimulation (Fig 1B). Serum and bile CXCL10 levels did not correlate with the SI values of the CD4+ T cells in response to both types of allostimulation (data not shown). CXCL10 levels in the serum were significantly correlated with those in the bile (Fig 2). Three patients experienced high CXCL10 levels in the bile, and these were far from the linear approximate curve. These patients suffered from severe acute cellular rejection that was refractory to steroid pulse.

DISCUSSION

We previously reported that the multiparametric MLR assay could optimize immunosuppressive therapy and provide a low incidence of acute rejection after LDLT [8,9]. In this study, CXCL10 levels in sera and bile after LDLT significantly correlated with the SI values of CD8+ T cells in response to allostimulation and with the CD25 expression of CD8+ T cell subsets in response to anti-donor stimulation in MLR assays, respectively. We previously demonstrated that the frequency of IFN-γ–producing cells was higher in the proliferated CD8+ T cell fraction than in the proliferated CD4+ T cell fraction [10]. This might be the reason why CXCL10, the protein induced by IFN-γ, correlates with the SI values and CD25 expression of the CD8+ T cell subsets but not the CD4+ T cell subsets. We also confirmed that the proliferating CD8+ CD25+ T cells in the MLR assay were cytotoxic to donor cells.

Taken together with the results that CXCL10 levels in bile correlated with the CD25 expression of CD8+ T cell subsets in response to anti-donor stimulation, the CXCL10 levels in the bile might reflect cytotoxicity to donor cells. Three patients who experienced high values of CXCL10 in the bile that were far from the linear approximate curve suffered from severe acute cellular rejection. Serum CXCL10 levels of these three patients were relatively low. This finding suggests that higher levels of CXCL10 in the bile might be produced in the liver.

The capacity to differentiate between immune activation toward infectious agents and allografts must be established for CXCL10 to be clinically applicable for predicting rejection. In fact, abnormal levels of CXCL10 were reportedly observed in the bodily fluids of individuals who were infected with intracellular bacteria and certain viruses [2,11–14]. Serum CXCL10 levels were significantly correlated with the number of cytomegalovirus (CMV) –positive cells per 50,000 peripheral white blood cells (data not shown). In contrast, CXCL10 levels in the bile did not correlate with the number of CMV-positive cells. This finding suggests that serum CXCL10 levels can also reflect systemic inflammation toward infectious agents.

In conclusion, measurements of CXCL10 levels in the bile could predict anti-donor cytotoxic T cell responses in liver transplant recipients. However, further investigation is required to determine the usefulness of CXCL10 in predicting the postoperative course.

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Alternative endocytosis pathway for productive entry of hepatitis C virus

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Previous studies have shown that hepatitis C virus (HCV) enters human hepatic cells through interaction with a series of cellular receptors, followed by clathrin-mediated, pH-dependent endocytosis. Here, we investigated the mechanisms of HCV entry into multiple HCV-permissive human hepatocyte-derived cells using trans-complemented HCV particles (HCVtcp). Knockdown of CD81 and claudin-1, or treatment with bafilomycin A1, reduced infection in Huh-7 and Huh7.5.1 cells, suggesting that HCV entered both cell types via receptor-mediated, pH-dependent endocytosis. Interestingly, knockdown of the clathrin heavy chain or dynamin-2 (Dyn2), as well as expression of the dominant-negative form of Dyn2, reduced infection of Huh-7 cells with HCVtcp, whereas infectious entry of HCVtcp into Huh7.5.1 cells was not impaired. Infection of Huh7.5.1 cells with culture-derived HCV (HCVcc) via a clathrin-independent pathway was also observed. Knockdown of caveolin-1, ADP-ribosylation factor 6 (Arf6), flotillin, p21-activated kinase 1 (PAK1) and the PAK1 effector C-terminal binding protein 1 of E1A had no inhibitory effects on HCVtcp infection into Huh7.5.1 cells, thus suggesting that the infectious entry pathway of HCV into Huh7.5.1 cells was not caveolae-mediated, or Arf6- and flotillin-mediated endocytosis and macropinocytosis, but rather may have occurred via an undefined endocytic pathway. Further analysis revealed that HCV entry was clathrin- and dynamin-dependent in ORL8c and HepCD81/miR122 cells, but productive entry of HCV was clathrin- and dynamin-independent in Hep3B/miR122 cells. Collectively, these data indicated that HCV entered different target cells through different entry routes.

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INTRODUCTION

Over 170 million people worldwide are chronically infected with hepatitis C virus (HCV), and are at risk of developing chronic hepatitis, cirrhosis and hepatocellular carcinoma (Hoofnagle, 2002). HCV is an enveloped virus belonging to the family *Flaviviridae*. Its genome is an uncapped 9.6 kb positive-stranded RNA consisting of the 5′-UTR, an ORF encoding viral proteins and the 3′-UTR (Suzuki *et al.*, 2007). A precursor polyprotein is further processed into structural proteins (core, E1, and E2), followed by p7 and non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B), by cellular and viral proteases.

Two supplementary figures are available with the online version of this paper.

Host–virus interactions are required during the initial steps of viral infection. Viruses enter the cells by various pathways, such as receptor-mediated endocytosis followed by pH-dependent or -independent fusion from endocytic compartments, or pH-independent fusion at the plasma membrane coupled with receptor-mediated signalling and coordinated disassembly of the actin cortex (Grove & Marsh, 2011). It was reported previously that CD81 (Bartosch *et al.*, 2003; McKeating *et al.*, 2004; Pileri *et al.*, 1998), scavenger receptor class B type I (SR-BI) (Bartosch *et al.*, 2003; Scarselli *et al.*, 2002), claudin-1 (Evans *et al.*, 2007; Liu *et al.*, 2009) and occludin (Benedicto *et al.*, 2009; Liu *et al.*, 2009; Ploss *et al.*, 2009) are critical molecules for HCV entry into cells. Recently, epidermal growth factor receptor and ephrin receptor type A2 were also identified as host cofactors for HCV entry, possibly by modulating interactions between CD81 and claudin-1 (Lupberger *et al.*,

2011). In addition, Niemann–Pick C1-like 1 (NPC1L1) cholesterol absorption receptor has been shown to play a role in HCV entry, probably at the fusion step (Sainz *et al.*, 2012).

Following receptor binding, HCV has been reported to enter cultured cells via clathrin-mediated endocytosis, the most common and best-characterized mode of endocytosis, following membrane fusion in early endosomes (Blanchard *et al.*, 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009) using retrovirus-based HCV pseudoparticles (HCVpp) and cell culture-produced HCV (HCVcc). Early steps in HCV infection, including the role of HCV glycoprotein heterodimers, receptor binding, internalization and pH-dependent endosomal fusion, have been at least in part mimicked by HCVpp. However, as HCVpp are generated in non-hepatic cells such as human embryo kidney 293T cells, it is likely that the cell-derived component(s) of HCVpp differ from those of HCVcc.

In the present study, we readdressed the HCV endocytosis pathway using trans-complemented HCV particles (HCVtcp) (Suzuki *et al.*, 2012), of which the packaged genome is a subgenomic replicon. HCVtcp, generated in Huh-7 or its derivative cell lines with two plasmids, are infectious, but support only single-round infection, thereby allowing us to examine infectious viral entry without the influence of reinfection. In addition, HCVtcp is useful for quantifying productive infection by measuring luciferase activity. Furthermore, it has been shown that the HCVtcp system is more relevant as a model of HCV infection than HCVpp (Suzuki *et al.*, 2012). Our results demonstrated conclusively that, in addition to the clathrin-mediated endocytosis pathway, HCV was capable of utilizing the clathrin- and dynamin-independent pathways for infectious entry of HCV into human liver-derived cells.

RESULTS

HCV entry depends on receptor-mediated, pH-dependent endocytosis

HCV has been shown to enter permissive cells through clathrin-mediated endocytosis and low pH-dependent fusion with endosomes mostly using HCVpp (Codran *et al.*, 2006; Meertens *et al.*, 2006; Trotard *et al.*, 2009), although some researchers have used HCVcc with limited cell lines (Blanchard *et al.*, 2006; Coller *et al.*, 2009). However, several distinct characteristics between HCVpp and HCVcc have recently been revealed with regard to morphogenesis and entry steps (Helle *et al.*, 2010; Sainz *et al.*, 2012; Suzuki *et al.*, 2012; Vieyres *et al.*, 2010). Therefore, in this study, we used HCVtcp, which exhibit similar characteristics to HCVcc when compared with HCVpp and support single-round infection (Suzuki *et al.*, 2012).

Initially, to determine whether receptor candidates such as CD81, claudin-1, occludin and SR-BI are essential for HCV entry into Huh-7 and Huh7.5.1 cells, we examined the knockdown effect of these molecules on HCVtcp infection. Knockdown of these receptors was confirmed by immunoblotting (Fig. 1a) and FACS analysis (Fig. 1b). It should be noted that the luciferase activity in Huh7.5.1 was approximately four times higher than that in Huh-7 cells when the same amount of inoculum was used for infection (Fig. S1, available in the online Supplementary Material), and knockdown did not affect cell viability (data not shown). Knockdown of CD81 and claudin-1 significantly reduced the infection of Huh-7 and Huh7.5.1 cells with HCVtcp derived from genotype 2a (Fig. 1c). Knockdown of occludin led to a moderate reduction in infection; however, only a marginal effect was observed in SR-BI knockdown in both Huh-7 and Huh7.5.1 cells (Fig. 1c), possibly due to the reduced requirement for SR-BI during virus entry by adaptive mutation in E2 (Grove *et al.*, 2008).

Next, to examine whether HCV entry was pH-dependent, Huh-7 and Huh7.5.1 cells were pretreated with bafilomycin A1, an inhibitor of vacuolar H^+-ATPases that impairs vesicle acidification, and then infected with HCVtcp. At 72 h post-infection, luciferase activity and cell viability were determined. Bafilomycin A1 inhibited HCVtcp infection in a dose-dependent manner without affecting cell viability in both Huh-7 and Huh7.5.1 cells (Fig. 2a, b). We also confirmed that treatment with bafilomycin A1 after HCVtcp infection had a minor effect on luciferase activity (Fig. 2c). These results indicated that the infectious route of HCVtcp into Huh-7 and Huh7.5.1 cells is receptor-mediated and involves pH-dependent endocytosis.

Knockdown of clathrin heavy chain (CHC) or dynamin-2 (Dyn2) reduces HCVtcp infection in Huh-7 cells, but not in Huh7.5.1 cells

Among the known pathways of pH-dependent viral endocytosis, clathrin-mediated dynamin-dependent endocytosis is a major endocytosis pathway. Chlorpromazine, an inhibitor of clathrin-dependent endocytosis, has been commonly used to study clathrin-mediated endocytosis; however, it exerts multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Therefore, we examined the HCV endocytosis pathway by knockdown of specific molecules required for the endocytosis pathway. CHC, a major structural protein in clathrin-coated vesicles, and Dyn2, a GTPase essential for clathrin-coated-pit scission from the plasma membrane, play important roles in the clathrin-mediated pathway. Another well-studied model of viral entry is caveolin-mediated endocytosis. The role of dynamin in both clathrin-mediated endocytosis and caveolae-dependent endocytosis has been established (Marsh & Helenius, 2006; Miaczynska & Stenmark, 2008). To examine the endocytosis pathways of HCV, small interfering RNAs (siRNAs) for CHC, Dyn2 and caveolin-1 (Cav1), or scrambled control siRNA, were transfected into Huh-7 or

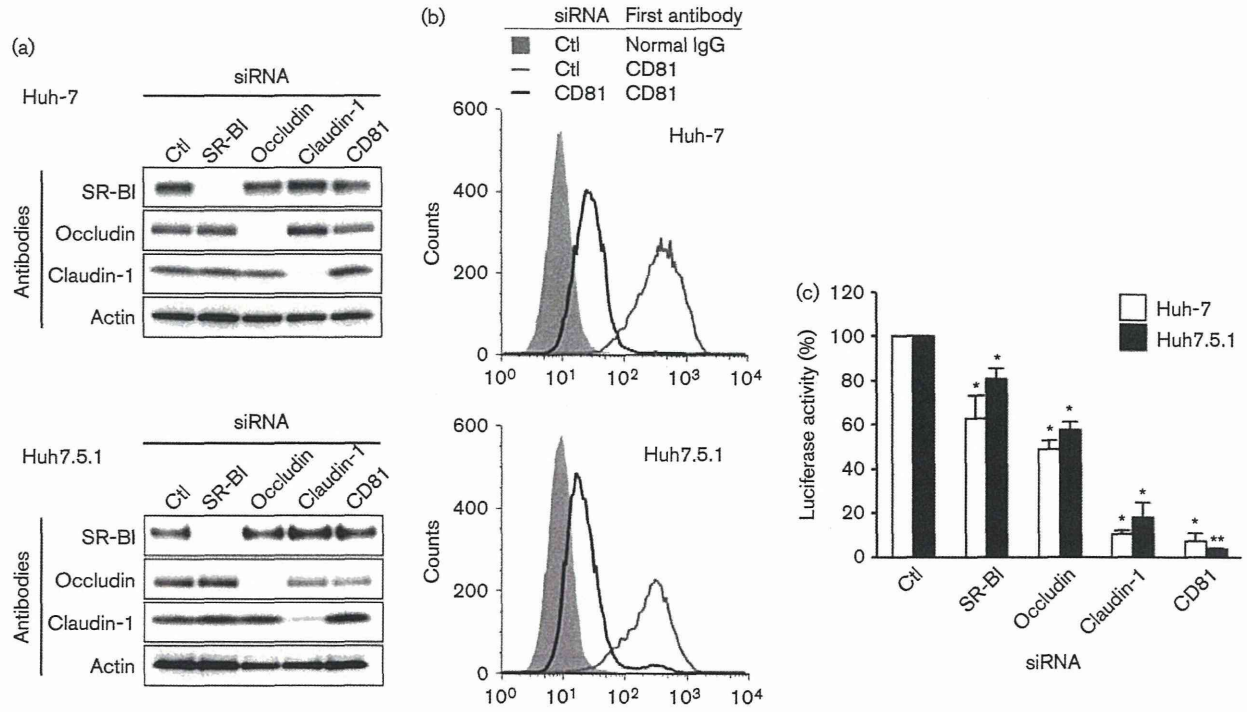

Fig. 1. Knockdown effect of receptor candidate molecules on HCV infection. (a) Huh-7 or Huh7.5.1 cells were transfected with the indicated small interfering RNAs (siRNA), harvested at 48 h post-transfection and the specific knockdown of each protein was verified by immunoblotting. (b) Huh-7 or Huh7.5.1 cells were transfected with CD81 or control siRNAs, harvested at 48 h post-transfection and the cell surface expression of CD81 was verified by FACS analysis. (c) Cells transfected with siRNA were infected with the same amount of HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was determined at 72 h post-infection and is expressed relative to the activity with control siRNA transfection. The value for control (Ctl) siRNA was set at 100 %. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

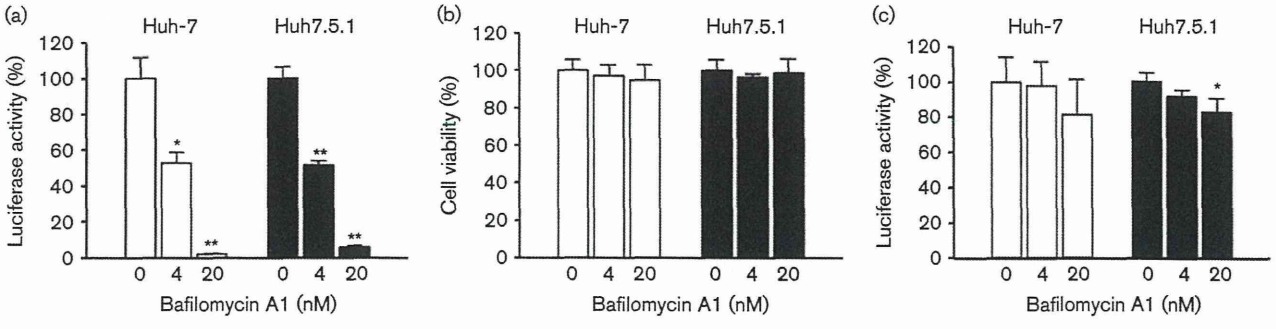

Fig. 2. Role of endosomal low pH in HCV infection. Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations and infected with HCVtcp. (a, b) Luciferase activity (a) and cell viability (b) were determined at 72 h post-infection, and expressed relative to amounts observed in controls. (c) Cells were treated with bafilomycin A1 for 1 h at the indicated concentrations 48 h after HCVtcp infection. Luciferase activity was determined at 10 h post-treatment and expressed relative to amounts observed in controls. Data represent the mean ± SD. Statistical differences between controls and indicated concentrations were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

Huh7.5.1 cells, followed by infection with HCVtcp. Expression of CHC, Dyn2 and Cav1 was downregulated by transfection of specific siRNAs (Fig. 3a, b), whereas expression of SR-BI, occludin, claudin-1 and CD81 was not reduced (Figs 3a and S2). As indicated in Fig. 3(c), luciferase activity from HCVtcp was significantly reduced by knockdown of CHC and Dyn2 in Huh-7 cells, but not in Huh7.5.1 cells. Knockdown of Cav1 showed no inhibitory effects on HCVtcp entry into either cell line. Dynamin-independent entry in Huh7.5.1 cells was also observed using HCVtcp derived from genotype 1b (data not shown). Knockdown of CHC or Dyn2 also reduced entry of HCVcc in Huh-7 cells, but had no inhibitory effects in Huh7.5.1 (Fig. 3d). To rule out the possibility of effects on CHC and Dyn2 knockdown on viral RNA replication, HCVtcp were also inoculated before siRNA transfection. Luciferase activity was not affected by knockdown of CHC or Dyn2 in either cell line, whereas marked inhibition was observed for phosphatidylinositol 4-kinase (PI4K) (Fig. 3e). These data suggested that HCV entry was clathrin-mediated and dynamin-dependent in Huh-7 cells, but productive entry of HCV was clathrin- and dynamin-independent in Huh7.5.1 cells.

Expression of the dominant-negative form of Dyn2 reduces HCV infection in Huh-7 cells, but not in Huh7.5.1 cells

We also examined the role of dynamin in infectious entry of HCV into Huh-7 and Huh7.5.1 cells by overexpression of the dominant-negative form of Dyn2 (Dyn-K44A), which

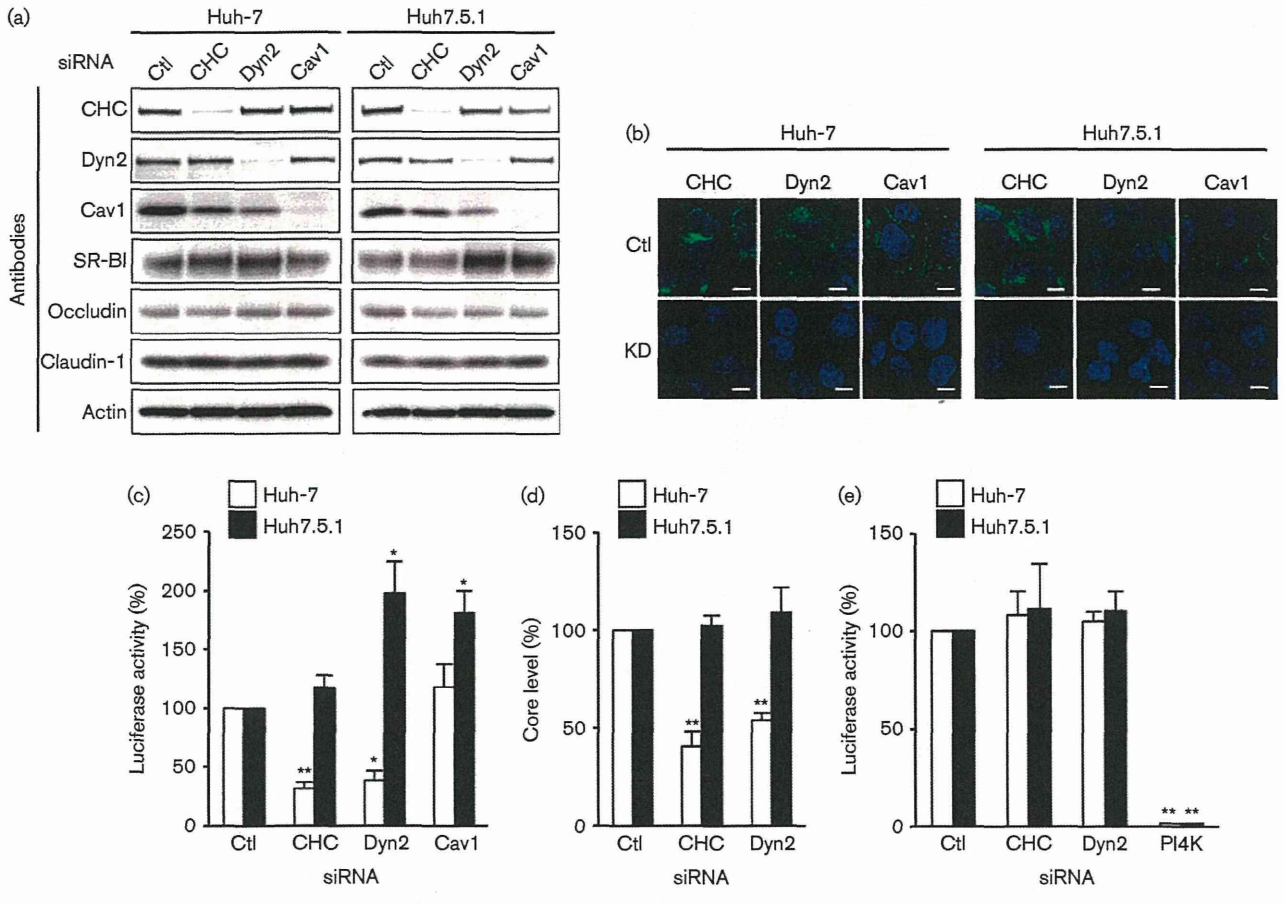

Fig. 3. Effects of CHC, Dyn2 and Cav1 knockdown on HCV infection. (a, b) Huh-7 cells or Huh7.5.1 cells were transfected with the indicated siRNAs and the specific knockdown (KD) of each protein was verified by immunoblotting (a) or immunostaining (b) at 48 h post-transfection. Bar, 50 μm. (c) Cells were transfected with the indicated siRNAs, followed by infection with HCVtcp at 48 h post-transfection. Firefly luciferase activity in the cells was subsequently determined at 3 days post-infection. The value for control (Ctl) siRNA was set at 100 %. Data represent the mean ± SD. (d) Cells were transfected with siRNA, followed by infection with HCVcc at 48 h post-transfection. Intracellular core levels were quantified at 24 h post-infection. The value for control siRNA was set at 100 %. Data represent the mean ± SD. (e) Cells were infected with HCVtcp, followed by transfection with the indicated siRNAs. Luciferase activity in the cells was subsequently determined at 2 days post-transfection. The value for control siRNA was set at 100 %. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

has been shown to effectively block clathrin-dependent and caveolar endocytosis (Damke *et al.*, 1995). Expression of haemagglutinin (HA)-tagged Dyn-K44A reduced the number of HCV-infected Huh-7 cells, but not Huh7.5.1 cells, as compared with WT HA-tagged Dyn2 (Dyn-WT), as shown in Fig. 4(a, b). Interestingly, internalization of transferrin, which is known to be mediated by clathrin-dependent endocytosis, was reduced in both Huh-7 and Huh7.5.1 cells expressing Dyn-K44A, whereas cells expressing Dyn-WT showed efficient endocytosis of transferrin (Fig. 4c, d). Collectively, these results suggested that dynamin participated in the internalization of HCV in Huh-7 cells, but was not absolutely required in Huh7.5.1 cells, although transferrin was taken up via dynamin-dependent endocytosis in both Huh-7 and Huh7.5.1 cells.

Flotillin-1 or the GTPase regulator associated with focal adhesion kinase 1 (GRAF1) play no major role during HCV infection of Huh7.5.1 cells

In order to dissect the major endocytosis pathways of HCVtcp in Huh7.5.1 cells, we investigated the role of alternative routes of HCV entry by siRNA knockdown. We silenced essential factors for the clathrin- or dynamin-independent pathways

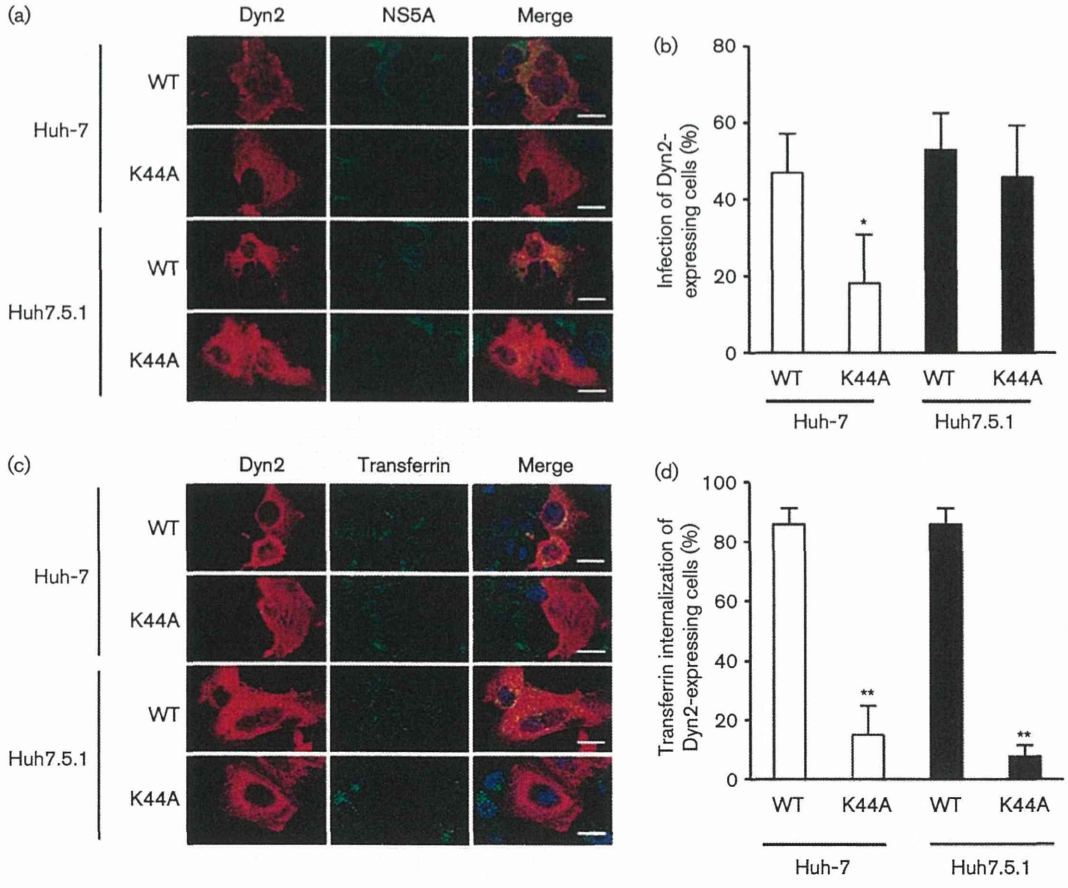

Fig. 4. Dynamin participates in the internalization of HCV in Huh-7 cells, but not in Huh7.5.1 cells. (a) Cells were transfected with HA-tagged WT Dyn2 (Dyn-WT) or dominant-negative Dyn2 (Dyn-K44A) expression plasmids. At 2 days post-transfection, cells were infected with HCVtcp, which possessed a subgenomic replicon without the luciferase gene. After 3 days, cells were fixed and HA-Dyn2 or HCV NS5A stained with anti-HA or anti-NS5A antibodies, respectively. Cell nuclei were counterstained with DAPI. Bar, 100 μm. (b) Data were quantified as the population of HCVtcp-infected cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean ± SD. (c) Cells were transfected with HA-tagged Dyn-WT or Dyn-K44A expression plasmids. At 2 days post-transfection, cells were incubated with Alexa Fluor-488 labelled transferrin at 37 °C in a 5 % CO_2 incubator. After 30 min of incubation, cells were washed, fixed and stained with anti-HA antibodies. Cell nuclei were counterstained with DAPI. Bar, 100 μm. (d) Data were quantified as the population of transferrin-internalized cells among HA-positive cells. At least 20 HA-positive cells were evaluated in triplicate experiments. Data represent the mean ± SD. Statistical differences between Dyn-WT and Dyn-K44A were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus Dyn-WT.

including flotillin-dependent endocytosis, ADP-ribosylation factor 6 (Arf6)-dependent endocytosis, clathrin-independent carrier/glycosylphosphatidylinositol-enriched early endosomal compartment (CLIC/GEEC) endocytic pathway and macropinocytosis in Huh7.5.1 cells. Flotillin-1 and Arf6 are indispensable components of the flotillin and Arf6 pathways, respectively. Knockdown of flotillin-1 or Arf6 had no inhibitory effects on HCVtcp infection in Huh7.5.1 cells (Fig. 5a). The CLIC/GEEC endocytic pathway has recently become better defined and is regulated by the GTPase regulator associated with focal adhesion kinase-1 (GRAF1). However, GRAF1 was not detected in Huh-7 or Huh7.5.1 cells (Fig. 5b); thus, it is unlikely that the CLIC/GEEC pathway was involved in HCV entry in Huh7.5.1 cells. In addition, knockdown of p21-activated kinase 1 (PAK1) and the PAK1 effector C-terminal binding protein 1 of E1A (CtBP1), which play important regulatory roles in the process of macropinocytosis, did not inhibit HCVtcp infection in Huh7.5.1 cells (Fig. 5c). Taken together, these results suggested that the entry of HCVtcp into Huh7.5.1 cells was not mediated mainly by flotillin-dependent endocytosis, Arf6-dependent endocytosis, the CLIC/GEEC endocytic pathway and macropinocytosis.

Clathrin-dependent and -independent pathways for HCV entry in other hepatic cells

We further examined the endocytosis pathways for HCV in non-Huh-7-related human liver-derived cell lines. Three HCVcc permissive hepatocellular carcinoma cell lines, Li23-derived ORL8c (Kato *et al.*, 2009), HepCD81/miR122 cells (HepG2/CD81 cells overexpressing miR122) and Hep3B/miR122 (Kambara *et al.*, 2012), were transfected with siRNA for CHC, Dyn2 or claudin-1, followed by infection with HCVtcp. Immunoblotting was performed in order to confirm knockdown of target proteins (Fig. 6a). Although knockdown of CHC or Dyn2 expression inhibited HCVtcp infection of ORL8c and HepCD81/miR122 cells, HCVtcp infection of Hep3B/miR122 cells was not affected (Fig. 6b), thus suggesting that productive entry of HCV is clathrin- and dynamin-independent in Hep3B/miR122 cells.

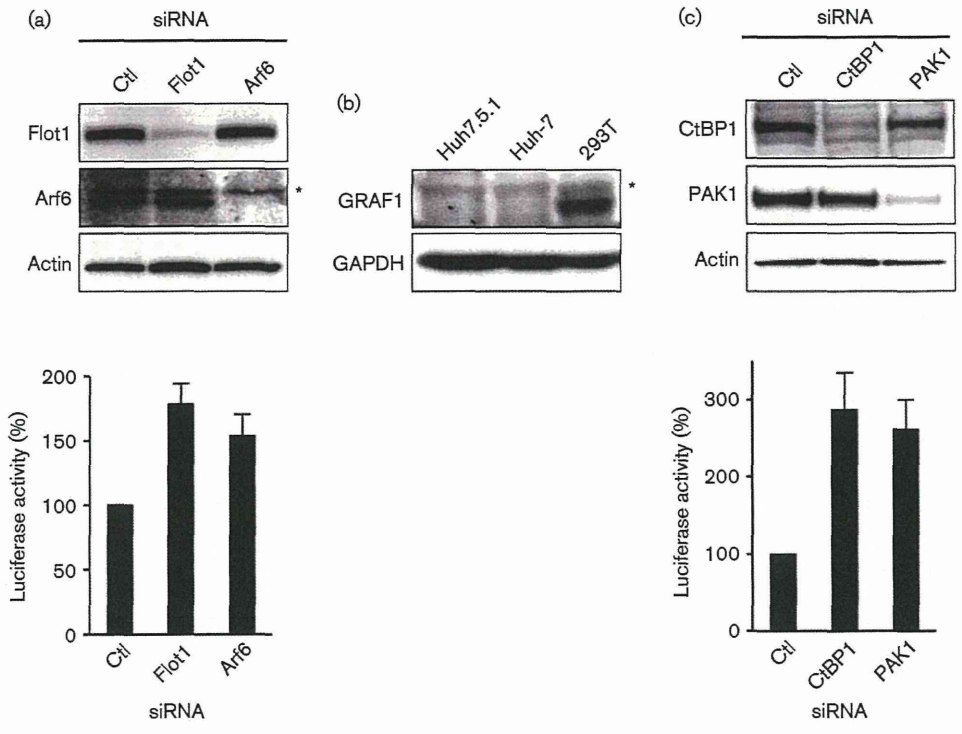

Fig. 5. Role of an alternative endocytosis pathway of HCV in Huh7.5.1 cells. (a) Huh7.5.1 cells were transfected with flotillin-1 (Flot1) or Arf6 siRNAs and specific knockdown of each protein was verified by immunoblotting (upper). Non-specific bands are marked with an asterisk. Cells transfected with siRNA were infected with HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection, Data represent the mean ± SD. (b) Expression of GRAF1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in Huh7.5.1, Huh-7 and 293T cells was analysed by immunoblotting. Non-specific bands are marked with an asterisk. (c) Huh7.5.1 cells were transfected with CtBP1 or PAK1 siRNA and specific knockdown of each protein was verified by immunoblotting (upper). Cells transfected with siRNA were infected with the HCVtcp. Luciferase activity (lower) was determined at 72 h post-infection and expressed relative to the amount observed in control (Ctl) siRNA transfection. Data represent the mean ± SD.

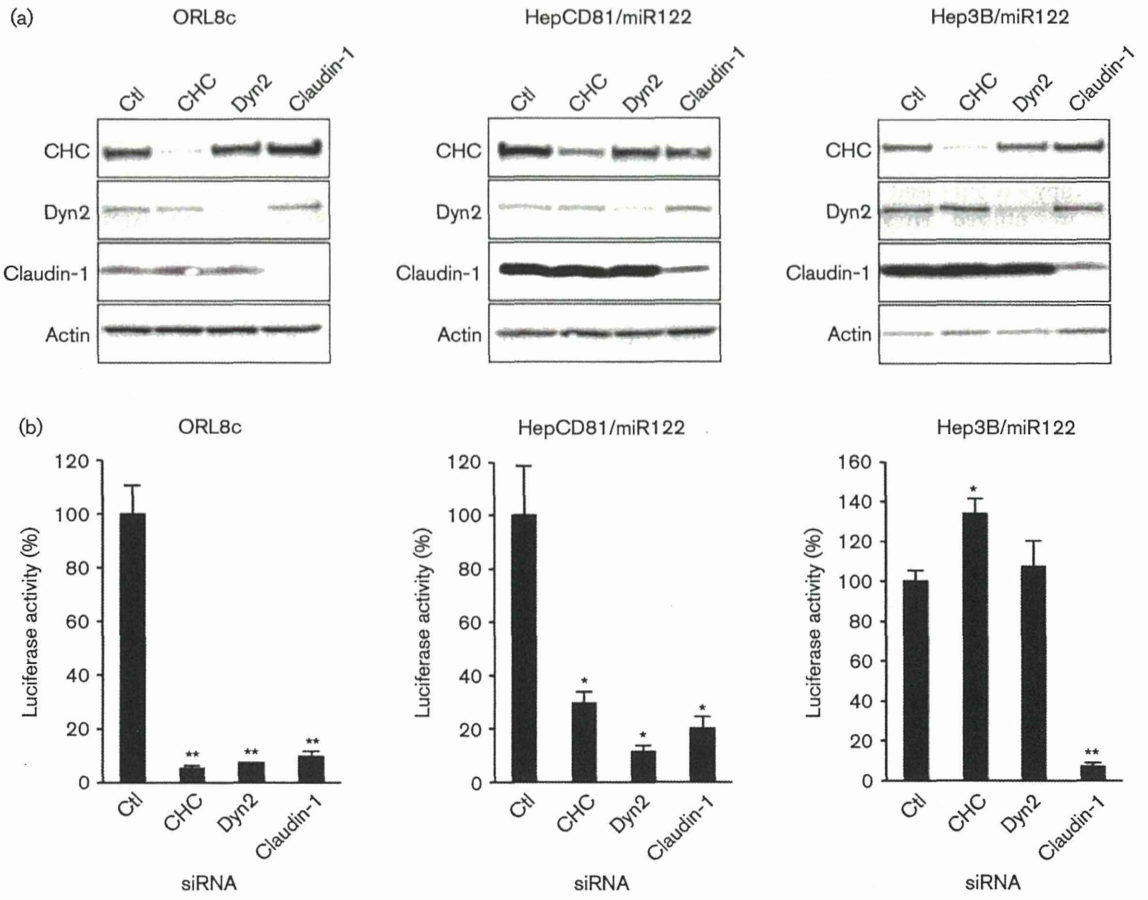

Fig. 6. Clathrin-dependent and -independent pathway of HCV entry in other HCV-permissive cells. The indicated cells were transfected with the indicated siRNAs and then infected with HCVtcp at 48 h post-transfection. (a) Specific knockdown of each protein was verified by immunoblotting. (b) Luciferase activity was determined at 72 h post-infection and expressed relative to the amount observed in the control (Ctl) siRNA transfection. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. **P*<0.05, ***P*<0.001 versus control.

In summary, we identified an alternative clathrin- and dynamin-independent entry pathway for HCV in at least two independent cell lines, Huh7.5.1 and Hep3B/miR122 cells, in addition to the previously reported clathrin- and dynamin-dependent pathway. These findings provided clues for understanding the molecular mechanisms of the endocytosis pathway for HCV infection.

DISCUSSION

Many viruses have been shown to utilize a number of different endocytic pathways to productively infect their hosts. Clathrin-dependent endocytosis would appear to be the most commonly used, but it is increasingly clear that a number of clathrin-independent endocytosis pathways are also used by several different viruses (Mercer *et al.*, 2010). In the case of HCV, it has been reported that viral entry is mediated by clathrin-dependent endocytosis (Blanchard *et al.*, 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009). In these papers, HCVpp was used at least in part for analysis of HCV entry pathway. However, recent reports have revealed several different characteristics between HCVpp and HCVcc.

Viral entry has been addressed primarily by pharmacologic inhibitor studies, immunofluorescence and electron microscopy, by transfection with dominant-negative constructs, and more recently by siRNA knockdown. Analysis of endocytosis pathways using pharmacological inhibitors has raised concerns about specificity. For example, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, has been shown to exert multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Methods for elucidating the viral endocytosis pathway by co-localization of virus particles with host factor also have limitations. Electron and

fluorescence microscopy, which require a high particle number, do not allow the differentiation of infectious and non-infectious particles. Infectious particles of HCV in the supernatant of infected cells appeared to represent only a small portion of secreted virus particles (Akazawa *et al.*, 2008) and it is unclear whether the viral particles observed by microscopy could lead to productive infection. Therefore, we utilized HCVtcp, which is useful for determining productive entry of the virus without reinfection, and a combination of siRNA knockdown and dominant-negative mutants for analysis of the productive route of infection. Although HCVcc is also utilized in analysis of productive entry, it cannot completely exclude the effects of reinfection by virus produced by infected cells. Reduction of HCVcc infection by knockdown of CHC and Dyn2 was moderate when compared with that of HCVtcp (Fig. 3c, d), thus suggesting slight effects due to reinfection in HCVcc.

The data presented here demonstrate for the first time to our knowledge that HCV is able to enter cells via dynamin-independent endocytosis in addition to the previously described classical clathrin- and dynamin-dependent pathway. First, knockdown of CHC and Dyn2 had no inhibitory effects on HCVtcp and HCVcc entry into Huh7.5.1 cells. Second, overexpression of dominant-negative Dyn2 had no inhibitory effects on HCVtcp in Huh7.5.1 cells. Finally, in addition to Huh7.5.1 cells, Hep3B/miR122 cells were also shown to be infected with HCV via clathrin- and dynamin-independent pathways. We further investigated the role of alternative minor routes of HCV entry into Huh7.5.1 cells; however, the productive endocytosis pathway could not be defined. It should be noted that inhibition of alternative endocytosis routes by siRNA led to an increase of luciferase activity (Figs 3c and 5a, c). This could be explained by the inhibition of a particular endocytosis pathway resulting in a compensatory increase in alternative endocytosis pathways (Damke *et al.*, 1995).

Although we confirmed an alternative endocytosis pathway for the productive entry of HCV, it is not clear why and how the two independent endocytosis pathways operate in different cell lines. SV40 can enter cells via caveolae-dependent (Norkin *et al.*, 2002; Pelkmans *et al.*, 2001) and -independent (Damm *et al.*, 2005) pathways. Influenza virus enters cells via clathrin-mediated endocytosis (Matlin *et al.*, 1981) in addition to non-clathrin-mediated, non-caveola-mediated internalization pathways (Sieczkarski & Whittaker, 2002b). Entry of dengue virus type 2 is clathrin-dependent in HeLa and C6/36 cells (Acosta *et al.*, 2008; Mosso *et al.*, 2008; van der Schaar *et al.*, 2008), and is clathrin-independent in Vero cells (Acosta *et al.*, 2009). Different receptor usage may determine the consequential route of entry. However, we did not observe any differences between Huh-7 and Huh7.5.1 cells in terms of knockdown effects of receptor candidate molecules on HCV infection, as shown in Fig. 1(c), although we cannot exclude the possibility that other undefined receptors are associated with viral entry. Huh7.5.1 cells were established by elimination of the HCV genome from replicon cells derived from Huh-7 cells (Blight *et al.*, 2002; Zhong *et al.*, 2005) and they exhibit more potent replication of HCV than the original Huh-7 cells. Further study showed that the increased permissiveness of cured cells results from a mutation in the retinoic acid-inducible gene I (Sumpter *et al.*, 2005), which impairs IFN signalling. In addition, it has been shown that cured cell lines express higher levels of miR122 than parental cells participating in the efficient propagation of HCVcc (Kambara *et al.*, 2012). As it is unclear whether these changes are the reason for a distinct endocytosis pathway, it will be of interest to explore these associations in further studies.

In conclusion, we confirmed an alternative clathrin-independent endocytosis pathway in HCV-permissive human hepatic-derived cells, in addition to the previously reported clathrin-dependent endocytosis pathway. This paper highlights the fact that clathrin- and dynamin-mediated endocytosis is the main route of HCV entry for Huh-7, HepCD81/miR122 and ORL8c cells, whilst clathrin and dynamin do not play a major role during the productive route of HCV infection in Huh7.5.1 and Hep3B/miR122 cells. Taken together, these studies suggest that different cell entry pathways for HCV infection may be utilized in different cell types, although further studies are necessary in order to understand this phenomenon.

METHODS

Cells. The human hepatocellular carcinoma cell lines Huh-7, Huh7.5.1, Hep3B/miR122 and HepG2/CD81, which overexpressed miR122 (Kambara *et al.*, 2012), were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries) containing non-essential amino acids, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10 % FBS. Li23-derived ORL8c cells (Kato *et al.*, 2009) were maintained in F12 medium and DMEM (1 : 1, v/v) supplemented with 1 % FBS, epidermal growth factor (50 ng ml^{-1}), insulin (10 μg ml^{-1}), hydrocortisone (0.36 μg ml^{-1}), transferrin (5 μg ml^{-1}), linoleic acid (5 μg ml^{-1}), selenium (20 ng ml^{-1}), prolactin (10 ng ml^{-1}), gentamicin (10 μg ml^{-1}), kanamycin monosulfate (0.2 mg ml^{-1}) and fungizone (0.5 μg ml^{-1}). All cell lines were cultured at 37 °C in a 5 % CO_2 incubator.

Preparation of viruses. HCVtcp and HCVcc derived from JFH-1 with adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) were generated as described previously (Suzuki *et al.*, 2012). For HepCD81/miR122 and ORL8c cells, HCVtcp containing the *Gaussia* luciferase (GLuc) reporter gene were used. To do this, plasmid pHH/SGR-JFH1/GLuc/NS3m carrying the bicistronic subgenomic HCV replicon containing the GLuc reporter gene and the NS3 adaptive mutation was constructed by replacement of the firefly luciferase (FLuc) gene of pHH/SGR-Luc containing the NS3 mutation (N1586D) (Suzuki *et al.*, 2012) with the GLuc gene of pCMV-GLuc (NEB).

Plasmids. HA-tagged Dyn2, a dominant-negative Dyn2 (K44A) in which Lys44 was replaced with Ala, was cloned into pcDNA3.1 as described previously (Kataoka *et al.*, 2012).

Gene silencing by siRNA. siRNAs were purchased from Sigma-Aldrich and were introduced into the cells at a final concentration of

30 nM using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. Target sequences of the siRNAs were: occludin (5′-GCAAGAUCACUAUGAGACA-3′), SR-BI (5′-GAGCUUUGGCCUUGGUCUA-3′), CD81 (5′-CUGUGAUCAUGAUCUUCGA-3′), CHC (5′-CUAGCUUUGCACAGUUUAA-3′), Dyn2 (5′-CCCUCAAGGAGGCGCUCAA-3′), Cav1 (5′-CCCUAAACACCUCAACGAU-3′), flotillin-1 (5′-CCUAUGACAUCGAGGUCAA-3′), Arf6 (5′-CAGUUCUUGGUAAAGUCCU-3′), CtBP1 (5′-GACUCGACGCUGUGCCACA-3′) and PAK1 (5′-GCAUCAAUUCCUGAAGAUU-3′). Target sequences of the siRNAs for claudin-1, PI4K and scrambled negative control were as described previously (Suzuki *et al.*, 2013).

Immunoblotting. Cells were washed with PBS and incubated with passive lysis buffer (Promega). Lysates were sonicated for 10 min and added to the same volume of 2× SDS-PAGE sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE and then transferred to PVDF membranes (Merck Millipore). After blocking, membranes were probed with primary antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) in accordance with the manufacturer's protocols.

Flow cytometry. Cultured cells detached by treatment with trypsin were incubated with anti-CD81 antibody or anti-mouse IgG antibody for 1 h at 4 °C. After being washed with PBS containing 0.1 % BSA, cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) for 1 h at 4 °C, washed repeatedly and resuspended in PBS. Analyses were performed using a FACSCalibur system (Becton Dickinson).

Reagents and antibodies. Bafilomycin A1 was obtained from Wako Pure Chemical Industries. Alexa Fluor 488-conjugated transferrin was obtained from Invitrogen. For immunoblotting, anti-SR-BI (NB400-104; Novus Biologicals), anti-occludin (71-1500; Invitrogen), anti-claudin-1 (51-9000; Invitrogen), anti-Dyn2 (ab3457; Abcam), anti-Cav1 (N-20; Santa Cruz Biotechnology), anti-flotillin (H-104; Santa Cruz Biotechnology), anti-Arf6 (ab77581; Abcam) and anti-PAK1 (2602; Cell Signaling Technology) rabbit polyclonal antibodies; anti-CD81 (JS-81; BD Biosciences), anti-β-actin (AC-15; Sigma-Aldrich), anti-CHC (23; BD Biosciences), anti-GRAF1 (SAB1400439; Sigma-Aldrich) and anti-glyceraldehyde 3-phosphate dehydrogenase (6C5; Merck Millipore) mouse mAb; and anti-CtBP1 goat polyclonal antibody (C-17; Santa Cruz Biotechnology) were used. For immunofluorescence staining, anti-CHC mAb (X22) and anti-HA rat polyclonal antibody (3F10) were obtained from Thermo Scientific and Roche Applied Science, respectively. Anti-NS5A antibody was a rabbit polyclonal antibody against synthetic peptides. Alexa Fluor 488- or 555-labelled secondary antibodies were obtained from Invitrogen.

DNA transfection. Cell monolayers were transfected with plasmid DNA using TransIT-LT1 transfection reagent (Mirus) in accordance with the manufacturer's instructions.

Treatment of cells with bafilomycin A1 and cell viability. Cells were preincubated with various concentrations of bafilomycin A1 for 60 min at 37 °C. Preincubated cells were then infected with HCVtcp. Cells treated with 0.1 % DMSO were used as controls. Cell viability was analysed by the Cell Titre-Glo Luminescent Cell Viability Assay (Promega).

Uptake of transferrin. Cells were grown on glass coverslips. After cells were transfected with HA-tagged Dyn2 expression plasmids, Alexa Fluor 488-conjugated transferrin at 20 μg ml^{-1} was added and incubated for 30 min. Cells were washed with PBS and fixed in 4 % paraformaldehyde.

Immunofluorescence analysis. Huh7.5.1 and Huh-7 cells were fixed with 4 % paraformaldehyde in PBS for 30 min, and were then blocked and permeabilized with 0.3 % Triton X-100 in a non-fat milk solution (Block Ace; Snow Brand Milk Products) for 60 min at room temperature. Samples were then incubated with anti-CHC, anti-Dyn2, anti-Cav1, anti-NS5A or anti-HA for 60 min at room temperature, washed three times with PBS, and then incubated with secondary antibodies for 60 min at room temperature. Finally, samples were washed three times with PBS, rinsed briefly in double-distilled H_2O and mounted with DAPI mounting medium. The signal was analysed using a Leica TCS SPE confocal microscope.

Luciferase assay. For quantification of FLuc activity in HCVtcp-infected cells, cells were lysed with passive lysis buffer (Promega) at 72 h post-infection. FLuc activity of the cells was determined using a luciferase assay system (Promega). For quantification of GLuc activity in supernatants of HCVtcp-infected cells, the *Renilla* Luciferase Assay System (Promega) was used. All luciferase assays were performed at least in triplicate.

Quantification of HCV core protein. HCV core protein was quantified using a highly sensitive enzyme immunoassay (Lumipulse G1200; Fujirebio) in accordance with the manufacturer's instructions.

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