Published in final edited form as:

Virus Res. 2012 September ; 168(1-2): 33–40. doi:10.1016/j.virusres.2012.06.009.

Mycophenolate Mofetil Inhibits Hepatitis C Virus Replication in Human Hepatic Cells

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Abstract

Hepatitis C virus (HCV) infection is the most common indication for liver transplantation and the major cause of graft failure. A widely used immunosuppressant, cyclosporine A (CsA), for people who receive organ transplantation, has been recognized to have the ability to inhibit HCV replication both *in vivo* and *in vitro*. In this study, we investigated the effects of several other immunosuppressants, including mycophenolate mofetil (MMF), rapamycin and FK506, on HCV replication in human hepatic cells. MMF treatment of hepatic cells before or during HCV infection significantly suppressed full cycle viral replication, as evidenced by decreased expression of HCV RNA, protein and production of infectious virus. In contrast, rapamycin and FK506 had little effect on HCV replication. Investigation of the mechanism(s) disclosed that the inhibition of HCV replication by MMF was mainly due to its depletion of guanosine, a purine nucleoside crucial for synthesis of guanosine triphosphate, which is required for HCV RNA replication. The supplement of exogenous guanosine could reverse most of anti-HCV effect of mycophenolate mofetil. These data indicate that MMF, through the depletion of guanosine, inhibits full cycle HCV JFH-1 replication in human hepatic cells. It is of interest to further determine whether MMF is indeed beneficial for HCV-infected transplant recipients in future clinical studies.

Keywords

HCV recurrence; liver transplantation; immunosuppressant; mycophenolate mofetil

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The authors have no conflicts of interest.

1 Introduction

Hepatitis C virus (HCV) infection is a common cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma, as well as the most common reason for liver transplantation worldwide. In the United States and Europe, end-stage liver disease associated with HCV infection has become the leading indication for liver transplantation (Limaye and Firpi, 2011). HCV recurrence is one of the risk factors associated with a worse outcome after transplantation. Persistence of HCV infection after transplantation is universal in immunosuppressed patients (Ciesek and Wedemeyer, 2011). The recurrence of HCV infection in post-transplantation occurs in at least 75%–80% of recipients, of whom 10%–21% develop fibrosis and cirrhosis (Chinnadurai et al., 2011). HCV-mediated liver injury in patients with HCV recurrence post-liver transplantation has been reported to follow a more progressive course compared to the non-immunosuppressed patients (McCashland, 2009; Zekry et al., 2004). Additionally, patients with recurrent chronic HCV infection develop higher viral load compared to pretransplant levels (McCashland, 2009). Such persistently high viral burden in post-transplantation may contribute to allograft damage. Long term follow-up of patients who underwent transplantation for end stage liver disease caused by HCV infection showed that patient and graft survival are decreased compared with HCV-negative patients (Rakela and Vargas, 2002). Therefore, the treatment of HCV infection in post-transplantation and the precise understanding of the interactions among HCV, anti-HCV drug, and immunosuppressive agents are extremely important for the long-term survival of transplant recipients (Gurusamy et al., 2009).

Cyclosporine (CsA) has been demonstrated to have the ability to suppress HCV replication both in vivo and in vitro (Inoue and Yoshida, 2005; Liu et al., 2011; Nakagawa et al., 2005). CsA-mediated anti-HCV activity is due to its ability to bind cyclophilins (Nakagawa et al., 2005). However, CsA is often associated with nonimmunologic adverse events, such as nephrotoxicity and neurotoxicity (Spencer et al., 1997). Among other immunosuppressive drugs used in transplantation, mycophenolate mofetil (MMF) has been proven to be effective and safe as an immunosuppressive agent in organ transplantations (Ramos-Casals and Font, 2005) and several autoimmune diseases (Chan et al., 2000; Langford et al., 2004). Because MMF lacks the nephrotoxicity associated with calcineurin inhibitors, CsA or FK506, it is often used to prevent rejection as a substitute or combined agent of CsA in liver and kidney transplantations (Pillai and Levitsky, 2009). MMF has anti-proliferative and immunosuppressive effects (Mitsui and Suzuki, 1969), as well as antiviral activity on some viruses, including Dengue virus, hepatitis B virus (HBV), avian reovirus, and human immunodeficiency virus-1 (HIV-1), Japanese encephalitis virus, Chikungunya virus (Diamond et al., 2002; Gong et al., 1999; Hossain et al., 2002; Khan et al., 2011; Sebastian et al., 2011). The clinical effects of MMF on HCV infection, however, are still controversial. Several studies showed that MMF treatment of rejection reduced the incidence of HCV recurrence after liver transplantation (Bahra et al., 2005; Jain et al., 2002; Kornberg et al., 2005; Manrique et al., 2008; Marubashi et al., 2009; Platz et al., 1998), while others reported no change or a slight increase in HCV viral load when MMF was used to treat HCV-infected transplant recipients (Fasola et al., 2002; Firpi et al., 2003; Ong et al., 1999; Rostaing et al., 2000; Zekry et al., 2004). Furthermore, to date, it has not been demonstrated whether MMF has any direct effect on HCV infection/replication *in vitro*, although a previous study (Henry et al., 2006) showed that mycophenolic acid (MPA), the active metabolite of MMF, has inhibitory effect on HCV replication in HCV replicon system. In this study, we used HCV JFH-1 infectious cell system that fully recapitulates viral entry, replication, and viral production (Wakita et al., 2005) to study the impact of MMF, as well as other immunosuppressive agents (rapamycin and FK506), on HCV replication in human hepatic cells. We also explored the mechanisms for the MMF action on HCV in human hepatic cells at cellular and molecular levels.

2 Methods

2.1 Reagents

MMF was purchased from Thermo Fisher Scientific Inc. (Barrington, IL). CsA, FK506, rapamycin, guanosine, dimethyl sulfoxide (DMSO), and Hoechst 33258 were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Mouse anti-HCV core and mouse anti-CD81 (JS-81) antibodies were obtained from Chiron Company (Emeryville, CA, USA) and BD Biosciences (San Jose, CA, USA), respectively. Alexa Fluor 488-conjugated goat anti-mouse IgG was purchased from Invitrogen (Carlsbad, CA, USA). CellTiter 96[®] AQueous One Solution Cell Proliferation Assay kit was purchased from Promega (Madison, WI, USA).

2.2 Cell culture and HCV JFH-1 infection

Hepatoma cell line Huh7 was kindly provided by Dr. Charles Rice (The Rockefeller University, New York, NY). Huh7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine at 5% CO₂. To generate infectious HCV JFH-1, *in vitro* transcribed genomic JFH-1 RNA was transfected into Huh7 cells as previously described (Wakita et al., 2005). Cell culture supernatant collected at day 10 post transfection was centrifuged, filtered (0.22 µm) and stored at -80 °C. HCV JFH-1 infection of Huh7 cells was performed at a MOI of 0.1 as previously described (Wakita et al., 2005).

2.3 The cytotoxic effect of MMF on the hepatic cells

The impact of MMF treatment on the viability of hepatic cells was demonstrated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Huh7 cells in 96-well plate were cultured for 24 h and then treated with MMF (0 to 32 µg/mL) for 72 h. For MTS assay, 20 µl of CellTiter 96[®] AQueous One Solution Reagent containing MTS and phenazine ethosulfate was added to each well of 96-well plate. Absorbance at 490 nm was measured at 4 h after addition of reagent.

2.4 Quantitative real-time RT-PCR

Total cellular RNA (1 µg) extracted from Huh7 cells was subjected to reverse transcription using the reverse transcription system from Promega (Madison, WI, USA). The real-time RT-PCR for quantification of intracellular or extracellular HCV RNA and target cellular mRNAs was performed as previously described (Ye et al., 2009). The oligonucleotide primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) and the primer sequences are listed in Table 1.

2.5 Quantitative real-time RT-PCR of microRNA

Total cellular RNA, including microRNA (miRNA) was extracted from cells and then reverse-transcribed to cDNA using miRNeasy Mini Kit and miScript Reverse Transcription Kit (Qiagen, Valencia, CA, USA), respectively. The real-time RT-PCR for quantification of a subset of miRNAs (miR-122, miR-196, miR-296, miR-351, miR-431, and miR-448) was carried out with miScript Primer Assays and miScript SYBR Green PCR Kit (Qiagen).

2.6 Immunofluorescence staining

The expression of HCV core protein was determined using a mouse anti-core antibody. Briefly, after washed with 1X Phosphate-Buffered Saline (PBS), the cells were fixed with PBS containing 4% paraformaldehyde and 4% sucrose for 20 min, permeabilized with PBS containing 0.5% Triton X-100 for 10min, treated with a blocking solution for 30 min. The cells were then incubated with the mouse anti-core antibody (1:100) for 1 h, and

subsequently incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200). The cell nuclei were stained with Hoechst 33258 and the cells were imaged using fluorescence microscope (Olympus, Japan). Quantification of core staining was performed with Image J software (<http://rsb.info.nih.gov>). Five random images of each experimental group (treated with or without MMF) were acquired and the fluorescence intensity of the staining positive (Green) was calculated using image J.

2.7 Titration of extracellular and intracellular HCV JFH-1

HCV JFH-1 infectivity from culture supernatants (extracellular) or cells (intracellular) treated with or without MMF was determined using Huh7 cells by end point dilution and immunofluorescence assay as previously described (Zhong et al., 2005). Briefly, for extracellular virus, culture supernatant was 10-fold serially diluted in DMEM-10% FBS and used to infect Huh7 cells in 96-well plates. HCV infectivity was examined 72 h post infection by immunofluorescence assay using mouse anti-core antibody. Viral infectivity is expressed as focus-forming units per milliliter of supernatant (FFU/mL). For intracellular virus, HCV JFH-1-infected cells were washed once with PBS and subsequently incubated with trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) for 5 min at 37°C. Cells were resuspended in DMEM-10% FBS and collected by centrifugation at 1,500 rpm for 3 min. Cell pellets were then resuspended in DMEM-10% FBS again and were lysed by four freeze-thaw cycles in dry ice and a 37°C water bath, respectively. Cell debris was pelleted by centrifugation at 4,000 rpm for 5 min. The supernatant was collected and used for HCV infectivity titration (Zhong et al., 2005).

2.8 HCV Pseudovirus and entry assay

HCV pseudovirus was generated by coexpression of an envelope-negative, luciferase-expressing HIV-1 genome (NL luc^+env^-) and HCV E1/E2 proteins from H77 subtype 1a isolate (Kolykhalov et al., 1997) as described previously (Bertaux and Dragic, 2006). Briefly, 293T cells were lipofected with a 1:2 ratio of NL luc^+env^- vector and HCV E1/E2 vector. Supernatant containing HCV pseudovirus was collected 36 h postlipofection and filtered (0.22 μ m). For HCV entry assay, Huh7 cells were pretreated with MMF at different concentrations or JS-81 at 10 μ g/mL at 37°C for 1 h, and then incubated with 200 μ l of HCV pseudovirus overnight. The cells were then washed and placed in fresh medium for another 36 h. Luciferase activity (relative lights units, RLU) was measured in cell lysates using luciferase kit (Promega) as previously described (Bertaux and Dragic, 2006).

2.9 Statistical analysis

Where appropriate, data were expressed as mean $\pm$ standard deviation (SD) of triplicate cultures. Statistical significance was assessed by Student *t* test. Statistical analyses were performed with Graphpad Instat Statistical Software. Statistical significance was defined as $P < 0.05$.

3 Results

3.1 MMF suppresses HCV JFH-1 replication

We first determined whether MMF has cytotoxicity on Huh7 cells. The cytotoxic concentration (CC50) of MMF on Huh7 cells is 32 μ g/mL. MMF at the concentrations of 1 μ g/mL or lower had minimal or no cytotoxicity (Fig. 1A). Therefore, for most subsequent experiments, we routinely used MMF at a dosage of 1 μ g/ml, which is lower than the typically used dose of ~10 μ g/mL for clinical immunosuppression.

Our data demonstrated that MMF treatment could inhibit HCV replication. As shown in Fig. 2A and 2B, MMF treatment of HCV-infected Huh7 cells significantly inhibited intracellular

and extracellular HCV RNA expression. The inhibition of HCV JFH-1 replication by MMF treatment was confirmed by the experiment examining HCV core protein expression. As demonstrated in Fig. 2C–2G, HCV core protein expression in MMF- or CsA-treated cells (Fig. 2D, 2F, 2G) was much lower than that in control cells (Fig. 2C, 2E, 2G). In contrast, FK506 or rapamycin treatment had little effect on HCV replication (Fig. 2A and 2B).

The inhibition of HCV JFH-1 replication by MMF is in a dose-dependent manner (Fig. 3). As shown in Fig. 3A and 3B, the MMF treatment inhibited the expression of intracellular and extracellular HCV RNA within the concentration range 0.1 µg/mL to 1.0 µg/mL. The effective concentration of MMF that inhibits 50% of intracellular HCV RNA replication (EC₅₀) is 0.297 µg/mL. To determine whether MMF treatment inhibits the production of infectious HCV, we also examined intracellular and extracellular HCV infectivity in cell cultures treated with or without MMF. Cell lysates and supernatant collected from JFH-1-infected Huh7 cultures treated with MMF had significant lower virus infectivity than those from the untreated cell cultures (Fig. 3C and 3D). Furthermore, to mimic *in vivo* situation where MMF treatment can be given to transplant recipients either before or after HCV infection, we treated Huh7 cells with MMF under different conditions. Huh7 cells were treated with MMF either 24 h prior to HCV infection, or simultaneously with infection, or 24 h postinfection (Fig. 3E–3H). Among the different conditions, pretreatment for 24 h had the highest inhibitory effect on HCV expression (80% HCV RNA and 90% infectious virus) (Fig. 3E–3H). MMF treatment of Huh7 cells during HCV infection also significantly suppressed HCV RNA expression as well as production of infectious virus (Fig. 3E–3H). Although MMF treatment after HCV infection had little effect on production of infectious virus (Fig. 3G and 3H), the treatment significantly decreased the levels of HCV RNA (Fig. 3E and 3F).

3.2 MMF does not affect HCV entry into human hepatic cells

To examine whether MMF affects HCV entry into human hepatic cells, we investigated whether MMF has the ability to modulate the expression of HCV entry receptors in Huh7 cells. Several cell surface molecules (CD81, claudin-1, LDLR, SB-R1, and occludin) have been proposed as HCV entry receptors or co-receptors. The expression of these HCV receptors in Huh7 cells was not affected by MMF treatment (Fig. 4A). To further confirm this finding, we used the pseudovirus enveloped with HCV E1/E2 proteins to evaluate the effect of MMF on HCV entry into Huh7 cells. The MMF treatment of Huh7 cells had little effect on pseudotyped HCV entry as determined by luciferase assay, whereas the mouse anti-CD81 (JS-81) could significantly inhibit HCV entry (Fig. 4B).

3.3 MMF does not affect type I IFN pathway and the expression of HCV-related miRNAs

Type I IFNs play a key role in host cell innate immunity against viral infections. Clinically, IFN-α is used for the treatment of HCV-infected patients. To investigate the possibility that MMF exerts anti-HCV activity through the activation of type I IFN pathway, we examined whether MMF treatment affects intracellular type I IFN expression in Huh7 cells. MMF treatment had little effect on IFN-α and IFN-β expression in Huh7 cells (Fig. 4C). We also analyzed the expression of IFN-stimulated genes in MMF-treated cells. MMF treatment had little effect on the expression of 2',5'-oligoadenylate synthetase (2'5'-OAS) and IFN-stimulated gene 56 (ISG56) (Fig. 4D). In addition, we examined whether MMF treatment modulates the expression of several intracellular miRNAs that have been identified to modulate HCV replication.(Jopling et al., 2005; Pedersen et al., 2007) As shown in Fig. 4E, MMF treatment had no significant effect on the expression of miR-122, miR-196, miR-296, miR-351, miR-431, and miR-448 in Huh7 cells.

3.4 Supplement of exogenous guanosine reduces MMF-mediated anti-HCV activity

MPA, the active metabolite of MMF, is an inhibitor of inosine monophosphate dehydrogenase (IMPDH) (Allison and Eugui, 2000). Hence, it is possible that MMF, through depletion of the intracellular pool of guanosine nucleotides, inhibits viral RNA synthesis. Therefore, we investigated the role of guanosine in the MMF action on HCV. The supplement of exogenous guanosine could reverse MMF-mediated HCV inhibition in a dose-dependent manner (Fig. 5). Guanosine could restore the most of inhibitory effect of MMF on HCV at both intracellular and extracellular levels (Fig. 5). However, guanosine was not able to completely reverse the anti-HCV effect of MMF. As shown in Fig. 5, the addition of guanosine only recovered the levels of HCV RNA, either intracellular or extracellular, to 60–70% of those in control cells (Fig. 5), even when its concentration was at a very high level (100uM) (Fig. 5)

4 Discussion

In this study, we investigated the effects of several immunosuppressants, including CsA, MMF, rapamycin and FK506, on HCV infection/replication in Huh7 cells. In addition to CsA, MMF was also demonstrated to have the ability to suppress HCV replication in Huh7 cells. This inhibitory effect of MMF on HCV was potent, as up to 90% of HCV replication was suppressed in the cells treated with MMF at a clinically relevant concentration (1 µg/mL). The liver transplant recipients receiving MMF had serum peak levels ranging from 0.6 µg/mL to 11.5 µg/mL (Bullingham et al., 1996; Patel and Akhlaghi, 2006). However, MMF at concentrations of higher than 1 µg/mL has cytotoxic effect (Fig. 1A). The selectivity index for MMF is about 108, which indicates that MMF can be considered as a potential anti-HCV drug.

MMF has been suggested as a possible antiviral agent because of its ribavirin-like effects (Allison and Eugui, 2000). Ribavirin and MPA (the metabolite of MMF) are two representative IMPDH inhibitors. Inhibition of IMPDH results in intracellular guanosine depletion, which is considered to be the antiviral mechanism of MMF against several viruses, including Dengue virus, HBV and HIV-1 as supplement by exogenous guanosine almost fully recovered viral replication (Diamond et al., 2002; Gong et al., 1999; Hossain et al., 2002; Khan et al., 2011; Sebastian et al., 2011; Takhampunya et al., 2006). Clinical studies of MMF effects on HCV have generated inconsistent data (Bahra et al., 2005; Fasola and Klintmalm, 2002; Firpi et al., 2003; Jain et al., 2002; Kornberg et al., 2005; Manrique et al., 2008; Marubashi et al., 2009; Ong et al., 1999; Platz et al., 1998; Rostaing et al., 2000; Zekry et al., 2004). In addition, little is known about the mechanism(s) of the MMF action on HCV. In this study, we showed for the first time that MMF treatment could directly inhibit HCV infection/replication *in vitro* and demonstrated that the depletion of intracellular guanosine nucleotide plays a role in MMF-mediated HCV inhibition, as the supplement with exogenous guanosine could largely reverse MMF-mediated HCV infection (Fig. 5). However, an early study (Henry et al., 2006) using MPA showed that HCV inhibition by MPA was not due to guanosine depletion. This discrepancy may be due to the fact that two different cell systems (HCV replicon system vs. full cycle infectious model) were used in the studies. HCV subgenomic replicon system only supports continuous HCV RNA genome replication without producing infectious virus particles. In our study, we used HCV JFH-1 infectious cell system that fully recapitulates viral entry, replication, and viral production (Wakita et al., 2005). It is known that HCV RNA synthesis prefers guanosine triphosphate (GTP) as the initiation nucleotide (Zhong et al., 2000) and high concentration of GTP is crucial to stimulate and initiate *de novo* RNA synthesis by HCV NS5B (Harrus et al.). An earlier study (Diamond et al., 2002) revealed that MPA treatment had much more remarkable influence on Dengue virus RNA replication at early stage of viral infection than at later stage of viral infection when the viral RNA is already at a steady level. Therefore, it

is possible that the GTP depletion has a significant influence on initiation of HCV RNA replication when its genomic RNA replication just starts, which explains why the MMF pretreatment resulted in a better inhibition of HCV than other treatment condition where MMF treatment was given after HCV infection, in which the HCV RNA has already been at a relatively stable level (Fig. 3E–3H). Nevertheless, supplement of guanosine was not able to completely reverse the inhibitory effect of MMF (Fig. 5), suggesting that other pathways involve in the anti-viral effect of MMF. Type I IFN pathway may involve in the antiviral activity of MPA because several studies have shown that MPA can act in synergy with IFN- α against different viruses (Henry et al., 2006; Khan et al., 2011). Although our study indicated that MMF treatment has little direct effect on expression of IFN- α/β and two ISGs (2'5'-OAS and ISG-56) (Fig. 4C and 4D), a recent study (Pan et al., 2012) demonstrated that MPA can induce expression of several other ISGs, including IRF-1, which has been shown to play an important role in anti-HCV effect through type I IFN pathway (Schoggins and Rice, 2012; Schoggins et al., 2011). Therefore, these findings suggest that an IMPDH-independent type I IFN pathway be another mechanism of MMF (MPA) inhibition of HCV replication.

To examine other mechanism(s) involved in the MMF action on HCV, we investigated whether MMF affects HCV entry into hepatic cells and intracellular innate immunity. We showed that MMF had little effect on the early steps (attachment, entry) of HCV infection, as MMF treatment did not affect the expression of HCV entry receptors as well as HCV entry into hepatic cells (Fig. 4A and 4B). In addition, MMF treatment of Huh7 cells did not alter the expression of several intracellular miRNAs (Fig. 4E) that have been identified to modulate HCV replication (Jopling et al., 2005; Pedersen et al., 2007).

Taken together, our study provides direct experiment evidence that MMF treatment of human hepatic cells inhibits full cycle HCV JFH-1 replication. Future clinical studies with well characterized subjects are necessary in order to determine whether MMF is indeed beneficial for HCV-infected transplant recipients.

Acknowledgments

We thank Drs. Charles M Rice (The Rockefeller University, New York, NY) and Takija Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for generously providing the Huh7 cell line and plasmid pJFH-1, respectively. We also thank Dr. Tatjana Dragic (Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York) for kindly providing HCV pseudovirus vectors (NLuc⁺env⁻ and pHCV E1/E2). This work was supported by the National Institutes of Health (grant numbers DA12815, DA22177, DA27550 to Dr. Wenzhe Ho). The sponsor has no role in study design, in data collection, analysis and interpretation, in manuscript writing, and in the decision to submit the article for publication.

Abbreviations

HCV	hepatitis C virus
CsA	cyclosporine
MMF	mycophenolate mofetil
JFH-1	Japanese fulminant hepatitis-1
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
miRNA	microRNA
IFN	interferon

2'5'-OAS	2',5'-oligoadenylate synthetase
ISG56	IFN-stimulated gene 56
MPA	mycophenolic acid
IMPDH	inosine monophosphate dehydrogenase
GTP	guanosine triphosphate

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Fig. 1. The cytotoxic effect of MMF on Huh7 cells

Huh7 cells were treated with various immunosuppressants (A) MMF; (B) CsA, Rapamycin and FK506 at indicated concentrations for 72 h. The cell viability was assessed by MTS assay. The results are the mean $\pm$ SD of three independent experiments (*, $P < 0.05$, **, $P < 0.01$, immunosuppressant treated vs control).

Fig. 2. Inhibition of HCV replication by MMF and CsA

Huh7 cells were cultured for 24 h in the presence of various immunosuppressants (1 μ g/mL for each) or vehicle controls (DMSO for FK506, rapamycin, MMF; alcohol for CsA) and then infected with HCV JFH-1 for 24 h. After infection, the cells were washed 5 times to remove input HCV and subsequently cultured in the presence of immunosuppressants for 72 h. The infected Huh7 cells and culture supernatant were collected for RNA analysis and immunofluorescence assay. (A, B) Effect of immunosuppressants on HCV RNA expression. The levels of intracellular (A) or extracellular (B) HCV RNA were determined by real-time RT-PCR. The levels of intracellular HCV RNA, with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, are expressed as the fold change versus control (defined as 1). The levels of extracellular HCV RNA are expressed as copies/mL. The results shown are the mean $\pm$ SD of triplicate cultures, representative of three experiments (**, $P < 0.01$, immunosuppressant treated vs vehicle treated). (C–F) Effect of MMF or CsA treatment on HCV core protein expression. The expression of HCV core protein was indicated (green) by immunofluorescence assay with a mouse anti-core antibody (magnification, 100 $\times$, scale bars, 100 μ m). The cell nuclei were stained with Hoechst 33258 (blue). (G) Image J analysis of average fluorescence intensity of five random images (HCV core protein, green) (a.u., arbitrary units). The data shown are the mean $\pm$ SD (* $p < 0.05$,

immunosuppressant treated vs vehicle treated, ⁺DMSO was used for dissolving MMF; ⁺⁺ Alcohol was used for dissolving CsA).

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Fig. 3. Inhibition of HCV replication and infectious virus production by MMF

(A–D) Dose-dependent effect of MMF. Huh7 cells were cultured for 24 h in the presence of MMF at shown concentrations or vehicle control (DMSO) and then infected with HCV JFH-1. After infection, the cells were cultured in the presence of MMF at shown concentrations for 72 h. The cells and culture supernatant were then collected for RNA analysis and HCV infectivity assay. (A, B) The levels of intracellular or extracellular HCV RNA were determined by real-time RT-PCR. The levels of intracellular HCV RNA (A), with normalization to GAPDH mRNA, are expressed as fold change versus control (defined as 1). The levels of extracellular HCV RNA (B) are expressed as copies/mL. (C, D) The levels of infectious virus (HCV infectivity) were determined by serial dilution and immunofluorescence. The intracellular (C) and extracellular (D) HCV infectivity is expressed as focus forming units per milliliter (FFU/mL). (E–H) MMF inhibits HCV JFH-1 replication under different conditions. Huh7 cells were cultured in the presence of MMF (1 μg/mL) or vehicle control (DMSO) for either 24 h prior to HCV JFH-1 infection, or simultaneously with infection, or 24 h postinfection. The infected cells were cultured in the presence of MMF for 72 h. Cell lysates and culture supernatant were then collected for RNA analysis and HCV infectivity assay. (E, F) The levels of intracellular (E) or extracellular (F) HCV RNA were determined by real-time RT-PCR. The levels of intracellular HCV RNA, with normalization to GAPDH mRNA, are expressed as fold change versus vehicle control (defined as 1). The levels of extracellular HCV RNA are expressed as copies/mL. (G, H) The levels of infectious virus (HCV infectivity) were determined by serial dilution and immunofluorescence. The intracellular (G) and extracellular (H) HCV infectivity is expressed as focus forming units per milliliter (FFU/mL). The results shown in the figure are the mean ± SD of triplicate cultures, representative of three experiments (*p<0.05, **P<0.01, MMF treated vs vehicle treated).

Fig. 4. MMF has little effect on HCV entry of hepatic cells, expression of type I IFNs, ISGs, and HCV-related microRNAs

(A) Effect of MMF on HCV receptor expression (CD81, claudin-1, LDLR, SB-RI, and occludin). Huh7 cells were cultured in the presence MMF (1 µg/mL) or vehicle control (DMSO) for 6 h or 24 h. Total cellular RNA was subjected to real-time RT-PCR for quantification of HCV receptor expression. The data are expressed as fold change versus control (defined as 1). (B) Effect of MMF on HCV pseudovirus entry into Huh7 cells. Huh7 cells were cultured in the presence of MMF or mouse anti-CD81 (JS-81) at indicated concentrations or vehicle control (DMSO) for 24 h, then infected with NLuc⁺env⁻ virus pseudotyped with HCV E1E2 glycoproteins in the presence of MMF or vehicle control. Luciferase activities (relative light units per mg cellular protein, RLU/mg protein) were measure at 36 h postinfection. The data are expressed as the RLU fold change versus vehicle control (defined as 1) (**p<0.01, JS-81 treated vs vehicle treated). (C–E) Effect of MMF on expression of type I IFNs (IFN-α/β) (C), 2',5'-oligoadenylate synthetase (2',5'-OAS) and IFN-stimulated gene 56 (ISG56) (D), and HCV-related miRNAs (miRNA-122, 196, 296, 351, 431, 448) (E). Huh7 cells were cultured in the presence MMF (1 µg/mL) or vehicle control (DMSO) for 6 h or 24 h. Total cellular RNA was subjected to real-time RT-PCR for quantification of cellular factor expression. The data are expressed as fold change versus control (defined as 1). The results shown are the mean ± SD of triplicate cultures, representative of three experiments.

Fig. 5. Supplement of exogenous guanosine reverses MMF-mediated inhibition of HCV replication

Huh7 cells were cultured for 24 h in the presence of MMF (1 μg/mL) and guanosine at shown concentrations and then infected with HCV JFH-1. After infection, the cells were cultured in the presence of MMF and guanosine for 72 h. The cells and culture supernatant were then collected for RNA analysis and HCV infectivity assay. (A, B) The levels of intracellular or extracellular HCV RNA were determined by real-time RT-PCR. The levels of intracellular HCV RNA (A), with normalization to GAPDH mRNA, are expressed as fold change versus vehicle control (defined as 1). The levels of extracellular HCV RNA (B) are expressed as copies/mL. (C, D) The levels of infectious virus within cells or in the culture supernatant were determined by serial dilution and immunofluorescence. The intracellular (C) and extracellular (D) HCV infectivity is expressed as focus forming units per milliliter (FFU/mL). The results shown are the mean ± SD of triplicate cultures, representative of three experiments (*p<0.05, **P<0.01, MMF treated alone vs MMF plus guanosine treated; #p<0.05, ##P<0.01, MMF plus guanosine treated vs Control).

Table 1**Primer Pairs for the Real-time RT-PCR**

Primer	Orientation	Sequence (5'-3')
HCV	Sense	RAYCACTCCCCTGTGAGGACC
	Antisense	TGRTGCACGGTCTACGAGACCTC
CD81	Sense	CGCCAAGGATGTGAAGCAGTTC
	Antisense	TCCCGGAGAAGAGGTCATCGAT
Claudin-1	Sense	GGCAGATCCAGTGCAAAGTC
	Antisense	TCTTCTGCACCTCATCGTCTT
LDLR	Sense	ACTGGTGTGAGAGGACCACC
	Antisense	CAAAGGAAGACGAGGAGCAC
SB-RI	Sense	GGTCCCTGTCATCTGCCAA
	Antisense	CTCCTTATCCTTTGAGCCCTTT
Occludin	Sense	AAGCAAGTGAAGGGATCTGC
	Antisense	GGGGTTATGGTCCAAAGTCA
IFN- α [†]	Sense	TTTCTCCTGCCTGAAGGACAG
	Antisense	GCTCATGATTTCTGCTCTGACA
IFN- β	Sense	GCCGCATTGACCATCTATGAGA
	Antisense	GAGATCTTCAGTTTCGGAGGTAAC
2',5'-OAS	Sense	AGAAGGCAGCTCACGAAACC
	Antisense	CCACCACCCAAGTTTCCTGTAHCV
ISG56	Sense	TTCGGAGAAAGGCATTAGA
	Antisense	TCCAGGGCTTCATTCATAT
GAPDH	Sense	GGTGGTCTCCTCTGACTTCAACA
	Antisense	GTTGCTGTAGCCAAATTCGTTGT

[†]The IFN- α primer pair matches 11 IFN- α subtypes: IFNA2, IFNA4, IFNA5, IFNA6, IFNA7, IFNA8, IFNA10, IFNA14, IFNA16, IFNA17, and IFNA21.